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Recombinant therapeutic single domain antibodies

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RECOMBINANT THERAPEUTIC SINGLE DOMAIN ANTIBODIES

FIELD OF THE INVENTION

The present invention provides single domain antibodies, more precisely heavy chain antibodies, having specificity to tumor necrosis factor alpha (TNF-alpha) and von Willebrand Factor (vWF) and their use in diagnosis and therapy. Such antibodies preferably have a framework sequence with high homology to the human framework sequences.

BACKGROUND TO THE INVENTION

Tumor necrosis factor alpha (TNF-alpha) is believed to play an important role in various diseases, for example in inflammatory diseases such as rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis. Both TNF-alpha and the receptors (CD120a, CD120b) have been studied in great detail. TNF-alpha in its bioactive form is a trimer and the groove formed by neighboring subunits is important for the cytokine-receptor interaction. Several strategies to antagonize the action of the cytokine have been developed and are currently used to treat various disease states.

A TNF inhibitor which has sufficient specificity and selectivity to TNF may be an efficient prophylactic or therapeutic pharmaceutical compound for preventing or treating inflammatory diseases. However, it is extremely difficult and a lengthy process to develop a small chemical entitiy (NCE) with sufficient potency and selectivity to such target sequence. Antibody-based therapeutics on the other hand have significant potential as drugs because they have exquisite specificity to their target and a low inherent toxicity. In addition, the development time can be reduced considerably when compared to the development of new chemical entities (NCE's). However, conventional antibodies are difficult to elicit against multimeric proteins where the receptor-binding domain of the ligand is embedded in a groove, as is the case with TNF-alpha. Heavy chain antibodies described in the invention which are derived from *Camelidae*, are known to have cavity-binding propensity (WO97/49805; Lauwereys et al, EMBO J. 17, 5312, 1998)). Therefore, such heavy chain antibodies are inherently suited to bind to receptor binding domains of such ligands as TNF. In addition, such antibodies are known to be stable over long periods of time, therefore increasing their shelf-life (Perez et al, Biochemistry, 40, 74, 2001). Furthermore, such heavy chain antibody fragments (coined VHH) can be produced 'en-masse' in fermentors using cheap expression systems compared to mammalian cell culture fermentation, such as yeast or other microorganisms (European patent number 0 698 097).

Upon damage to a blood vessel, subendothelial structures are exposed that mediate platelet adhesion through interaction with von Willebrand factor (vWF). vWF forms a bridge between collagen within the damaged vessel wall and the platelet receptor glycoprotein Ib (gpIb), an interaction especially important under high shear conditions, leading to the formation of a haemostatic plug and thus preventing excessive bleeding (Bennett S, Thromb Haemost (2001) Mar;**85**(3):395-400). During normal haemostasis, these processes lead to wound healing of the damaged blood vessel wall. In pathological conditions however, excessive platelet function may lead to thrombus formation. The vWF subunit is composed of several homologues domains each covering different functions. vWF interacts through its A3 domain with fibrillar collagen fibers and through its A1 domain with the platelet receptor gpIb. Under normal conditions platelets and vWF do not interact. However, when vWF is bound to collagen at high shear rate, it is believed to undergo a conformational change allowing its binding with the platelet receptor gpIb. This reversible adhesion allows platelets to roll over the damaged area, which is then followed by a firm adhesion through the collagen receptors on the platelets (gpIa/IIa, gpVI, gpIV, p65, TIIICBP) resulting in platelet activation. This leads to activation of the gpIIb/IIIa receptor, fibrinogen binding, and finally to platelet aggregation.

Abciximab (Chimeric 7E3 Fab; ReoPro), the Fab fragment of the mouse human chimeric antibody 7E3, which inhibits ligand binding to the platelet gpIIb/IIIa receptor was approved for human use as adjunctive therapy to prevent ischemic complications of percutaneous coronary interventions in December 1994. The principle safety issue with gp IIb/IIIa inhibitors is the risk of bleeding, as the potent anti-platelet effect of these drugs may adversely affect haemostasis. In addition, antagonists of gp IIb/IIIa may increase the risk of thrombocytopenia (Waldmann TA *et al*, Hematology (2000): 394 to 408).

A murine anti-human vWF monoclonal antibody, AJvW-2, was developed that inhibited the interaction between platelet glycoprotein Ib (gpIb) and von Willebrand factor (vWF) during the ristocetin- and botrocetin- induced aggregation of human platelets (PCT application number WO 00/10601). AJvW-2 inhibited the high shear stress induced aggregation of human platelets, but had no effect on low shear stress induced platelet aggregation.

The effects of F(ab) fragments of a murine antibody raised against the A3 domain of human vWF, 82D6A3, in baboons are disclosed in PCT application number WO 02/051351, and Dongmei Wu *et al*, Hemostasis, thrombosis and vascular biology, 2002, 99: 3623-3628.

A non-humanised murine monoclonal antibody raised against vWF A3 domain is disclosed in US patent application number 2002/0028204 A1 and US patent number 6,280,731.

Antibody 6B4 is a monoclonal antibody (MoAb) raised against purified human gpIb. MoAb 6B4 inhibits both ristocetin- and botrocetin-induced, vWF-dependent human platelet agglutination. MoAb 6B4 furthermore blocks shear-induced adhesion of human platelets to collagen I. When injected into baboons, intact IgG and its F(ab')(2) fragments caused almost immediate thrombocytopenia, due to the bivalency of F(ab')(2) which mediates platelet crosslinking, or Fc:Fc receptor interactions which mediate activation of platelet aggregation (Cauwenberghs N. *et al*, Arteriosclerosis, Thrombosis and Vascular biology, 2000, 20: 1347 and see, for example, Cadroy Y et al, Blood, 1994, 83: 3218-3224, Becker BH et al, blood, 1989, 74: 690-694, Ravanat C. et al, thromb. Haemost. 1999 , 82 : 528a abstract). Platelet deposition onto collagen-rich bovine pericardium was inhibited when Fab fragments were injected into the baboons before a thrombus was generated. However, when the Fab fragments were injected after a thrombus was allowed to form, no inhibition of further thrombosis was observed. The yields of expression of said Fab molecules are very low and the method of production is very labor intensive.

The use of antibodies derived from sources such as mouse, sheep, goat, rabbit etc., and humanised derivatives thereof as a treatment for conditions which require a modulation of platelet aggregation, is problematic for several reasons. Traditional antibodies are not stable at room temperature, and have to be refrigerated for preparation and storage, requiring necessary refrigerated laboratory equipment, storage and transport, which contribute towards time and expense. Refrigeration is sometimes not feasible in developing countries. Furthermore, the manufacture or small-scale production of said antibodies is expensive because the mammalian cellular systems necessary for the expression of intact and active antibodies require high levels of support in terms of time and equipment, and yields are very low. Furthermore the large size of said antibodies is not suited to interaction with macromolecules which are starting, or are in the process of aggregating, such as those involved in platelet aggregation, due to the restricted space available between molecules in an aggregating complex. For example, it has been shown that not all A3 domains are accessible to conventional antibodies in *in vivo* experiments (Dongmei WU, Blood, 2002, 99, 3623 to 3628). Furthermore the large size of conventional antibodies, would restrict tissue penetration, for example, during platelet aggregation at the site of a damaged vessel wall. Furthermore, traditional antibodies have a binding activity which depends upon pH, and hence are unsuitable for use in environments outside the usual physiological pH range such as, for example, in treating gastric bleeding,

gastric surgery. Furthermore, traditional antibodies are unstable at low or high pH and hence are not suitable for oral administration. However, it has been demonstrated that camelid antibodies resist harsh conditions, such as extreme pH, denaturing reagents and high temperatures (Ewert S et al, Biochemistry 2002 Mar 19; 41(11):3628-36), so making them suitable for delivery by oral administration. Furthermore, traditional antibodies have a binding activity, which depends upon temperature, and hence are unsuitable for use in assays or kits performed at temperatures outside biologically active-temperature ranges (*e.g.* 37 ± 20°C).

Polypeptide therapeutics and in particular antibody-based therapeutics have significant potential as drugs because they have exquisite specificity to their target and a low inherent toxicity. However, it is known by the skilled addressee that an antibody which been obtained for a therapeutically useful target requires additional modification in order to prepare it for human therapy, so as to avoid an unwanted immunological reaction in a human individual upon administration thereto. The modification process is commonly termed "humanization". It is known by the skilled artisan that antibodies raised in species, other than in humans, require humanization to render the antibody therapeutically useful in humans. There is a need for a method for producing antibodies which avoids the requirement for substantial humanization, or which completely obviates the need for humanization. There is a need for a new class of antibodies which have defined framework regions or amino acid residues and which can be administered to a human subject without the requirement for substantial humanization, or the need for humanization at all.

Another important drawback of conventional antibodies is that they are complex, large molecules and therefore relatively unstable, and they are sensitive to breakdown by proteases. This means that conventional antibody drugs cannot be administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation because they are not resistant to the low pH at these sites, the action of proteases at these sites and in the blood and/or because of their large size. They have to be administered by injection (intravenously, subcutaneously, etc.) to overcome some of these problems. Administration by injection requires specialist training in order to use a hypodermic syringe or needle correctly and safely. It further requires sterile equipment, a liquid formulation of the therapeutic polypeptide, vial packing of said polypeptide in a sterile and stable form and, of the subject, a suitable site for entry of the needle. Furthermore, subjects commonly experience physical and psychological stress prior to and upon receiving an injection. Therefore, there is need for a method for the delivery of therapeutic polypeptides which

avoids the need for injection which is not only cost/time saving, but which would also be more convenient and more comfortable for the subject.

THE AIMS OF THE PRESENT INVENTION

It is an aim of the present invention to provide a new class of *Camelidae* heavy chain variable domain antibodies (VHHs) which can be administered to a human subject without the requirement for substantial humanization, or the need for humanization at all. It is a further aim of the present invention to provide methods for providing said VHHs.

A further aim of the present invention is to provide polypeptides which bind to TNF-alpha, homologues of said polypeptides, and/or functional portions of said polypeptides, in a manner such that upon binding the biological activity of TNF-alpha is modified. Such polypeptides preferably bind into the receptor-binding groove of TNF-alpha. Such ligands are preferably single domain antibodies, such as heavy chain variable domain antibodies derived from *Camelidae*

A further aim of the present invention is to provide polypeptides, such as heavy chain variable domain antibodies derived from *Camelidae*, directed to vWF, vWF A1 domain, vWF A3 domain, gpIb, gpIa/IIa, or collagen, homologues of said polypeptides, and/or functional portions of said polypeptides, for the treatment for conditions which require a modulation of platelet aggregation and which overcomes the problems of the prior art. It is a further aim to provide methods of production of said polypeptides, methods and kits for screening for agents that modulate platelet aggregation and kits for the diagnosis of diseases related to platelet aggregation.

It is a further aim of the invention to provide a method of administering protein therapeutic molecules orally, sublingually, topically, nasally, vaginally, rectally or by inhalation which overcomes the problems of the prior art. It is a further aim to provide said therapeutic molecules.

DETAILED DESCRIPTION

The present invention relates to VHH molecules - derived from antibodies raised in *Camelidae* species, for example in camel, dromedary, alpaca and guanaco - and their use in the biological modulation of drug targets. VHH molecules are about 10x smaller than IgG molecules. They are single polypeptides and very stable, resisting extreme pH and

temperature conditions. Moreover, they are resistant to the action of proteases which is not the case for conventional antibodies.

The present invention further relates to the finding that a new class of camelid single domain heavy chain antibodies (VHHs) have human-like sequences. Surprisingly, the inventors have found that VHHs carrying an amino acid from the group consisting of glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tyrosine, tryptophan, methionine, serine, threonine, asparagine, or glutamine at position 45, such as, for example, L45 and a tryptophan at position 103, according to the Kabat numbering, have more human sequence character than other VHHs. The VHHs sequences represented by SEQ ID NO: 1 and SEQ ID NO: 13 which bind to von Willebrand factor (vWF) and tumor necrosis factor alpha (TNF-alpha) respectively, belong to this new class of VHH peptides. As such, peptides belonging to this new class show a high amino acid sequence homology to human VH framework regions and said peptides might be administered to patients directly without expectation of an unwanted immune response therefrom, and without the burden of further humanization. Furthermore, VHH molecules derived from *Camelidae* antibodies are among the smallest intact antigen-binding domains known (approximately 15 kDa, or 10 times smaller than a conventional IgG) are hence are well suited towards delivery to dense tissues and/or tumors. SEQ ID NOs: 1 and 13 and other VHHs of this class and their derivatives not only possess the advantageous characteristics of conventional antibodies, such as low toxicity and high selectivity, but they also exhibit additional properties. They are more soluble, meaning they may be stored and/or administered in higher concentrations compared with conventional antibodies. They are stable at room temperature meaning they may be prepared, stored and/or transported without the use of refrigeration equipment, conveying a cost, time and environmental savings (see for example, Example 11). Other advantageous characteristics as compared to conventional antibodies include short half-life in the circulation which may be modulated according to the invention by, for example, Fc coupling, VHH coupling (bivalent VHHs) or by pegylation (see for example, Figure 9). The polypeptides also retain binding activity at a pH and temperature outside those of usual physiological ranges, which means they may be useful in situations of extreme pH and temperature. The polypeptides also exhibit a prolonged stability at extremes of pH, meaning they would be suitable for delivery by oral administration. The polypeptides may be cost-effectively produced through fermentation in convenient recombinant host organisms such as *Escherichia coli* and yeast; unlike conventional antibodies which also require expensive mammalian cell culture facilities, achievable levels of expression are high. Examples of yields of the polypeptides of the present invention are 1 to 10 mg/ml (*E. coli*) and up to 1g/l (yeast). The polypeptides also

exhibit high binding affinity for a broad range of different antigen types, and ability to bind to epitopes not recognised by conventional antibodies; for example they display long CDR-based loop structures with the potential to penetrate into cavities and exhibit enzyme function inhibition. Furthermore, since binding often occurs through the CDR3 loop only, it is envisaged that peptides derived from CDR3 might be used therapeutically (Desmyter *et al.*, *J Biol Chem*, 2001, 276: 26285-90). The polypeptides are also able to retain full binding capacity as fusion protein with an enzyme or toxin. Thus the polypeptides represented by SEQ ID NOs: 1 and 13 and other VHHs of this class and their derivatives, homologues or functional portions thereof provide a considerable cost and time saving, particularly as they may be expected to be administered to patients directly without an unwanted immune response therefrom, and without the burden further humanization. Thus, a patient in need of said polypeptides would encounter fewer of the problems associated with conventional agents.

The present invention further relates to a VHH for use in treating, preventing and/or alleviating the symptoms of inflammatory diseases. A non-limiting example of a therapeutic target against which the VHHs of the invention may be used is TNF-alpha, which is involved in inflammatory processes.The blocking of TNF-alpha action can have an anti-inflammatory effect, which is highly desirable in certain disease states such as, for example, Crohn's disease. Current therapy consists of intravenous administration of anti-TNF-alpha antibodies. Our Examples demonstrate VHHs according to the invention which bind TNF-alpha and moreover, block its binding to the TNF receptor. Oral delivery of these anti-TNF-alpha VHH results in the delivery of such molecules in an active form in the colon at sites that are affected by the disease. These sites are highly inflamed and contain TNF-alpha producing cells. These anti-TNF-alpha VHH can neutralise the TNF-alpha locally, avoiding distribution throughout the whole body and thus limiting negative side-effects. Genetically modified microorganisms such as *Micrococcus lactis* are able to secrete antibody fragments. Such modified microorganisms can be used as vehicles for local production and delivery of antibody fragments in the intestine. By using a strain which produces a TNF-alpha neutralizing antibody fragment, inflammatory bowel disease could be treated. Another aspect of the invention is one or more VHHs specific for TNF-alpha for use in the treatment, prevention and/or alleviation of diseases relating to inflammatory processes, wherein said VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating diseases relating to inflammatory processes, comprising administering to a subject a VHH specific for an antigen related to the disease orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. The anti-TNF-

alpha VHHs of the present invention may be derived from the new class of VHHs described above, or may be derived from any of the other classes of VHHs, including the major class of VHH as the Examples illustrate.

One aspect of the present invention relates to polypeptides corresponding to SEQ ID NOs: 11 to 23, derived from llama heavy chain antibodies (VHHs), which bind to TNF-alpha.

The aspect of the invention relating to anti-TNF-alpha VHH polypeptides is not limited to polypeptides represented by SEQ ID NOs: 11 to 23, but may be extended to encompass polypeptides comprising camelidae antibodies of any class directed towards TNF-alpha. These polypeptides include the full length camelid antibodies, namely Fc and VHH domains. These polypeptides are known herein as "other anti-TNF-alpha polypeptides".

It is well known in the art that delivery of a therapeutic dose of a compound capable of binding a target molecule in the gut (such as TNF) should be able to pass through the gastric environment without being inactivated. Heavy chain antibody domains derived from *Camelidae* are known to have remarkable stability under extreme conditions, such as acidic pH (REF EP1134231). As known by persons skilled in the art, once in possession of said VHHs, formulation technology may be applied to release a maximum amount of VHH's in the right location (in the stomach, in the colon, etc.). This method of delivery is important for treating, prevent and/or alleviate the symptoms of disease whose targets that are located in the gut system. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of a disease requiring the delivery of a therapeutic compound that is able pass through the gastric environment without being inactivated, by orally administering to a subject a VHH specific for an antigen related to the disease.

Another aspect of the present invention, referred to herein as an "aggregation-related" aspect relates to the finding that polypeptides C37, T76, 2L-34 and 4L-16 represented by SEQ ID NOs: 1, 2, 8 and 9 as in Table 1 derived from a monoclonal llama antibody, binds to the A3 domain of vWF. It also relates to the finding that the polypeptides A50, A38, I53, M53 and Z29, represented by SEQ ID NOs: 3 to 7 derived from monoclonal llama antibodies, bind to the A1 domain of vWF. The aggregation-related aspect of the present invention further relates to *Camelidae* antibodies directed towards to vWF, vWF A1 or A3

domains, gpIb, gpIa/IIa, or collagen. The aggregation-related aspect of the present invention is not limited to VHHs belonging the new class described above but includes VHHs belonging to more than one class of VHH as the Examples illustrate.

According to the "aggregation-related" aspect of the invention, polypeptides corresponding to SEQ ID NOs: 1 to 9 are derived from llama heavy chain antibodies. Said polypeptides have a unique structure that consists of a single variable domain (referred to as the VHH by persons skilled in the art). VHH molecules derived from camelid antibodies are among the smallest intact antigen-binding domains known (approximately 15 kDa, or 10 times smaller than a conventional IgG) are hence are well suited towards delivery to dense tissues and/or tumors, and for accessing the limited space between macromolecules participating in or starting the process of platelet aggregation. SEQ ID NOs: 1 to 9 and their derivatives not only possess the advantageous characteristics of conventional antibodies, such as low toxicity and high selectivity, but they also exhibit additional properties. They are more soluble, meaning they may be stored and/or administered in higher concentrations compared with conventional antibodies. They are stable at room temperature meaning they may be prepared, stored and/or transported without the use of refrigeration equipment, conveying a cost, time and environmental savings (see, for example, Experiment 11). Other advantageous characteristics as compared to conventional antibodies include short half-life in the circulation which may be modulated according to the invention by, for example, Fc coupling, VHH coupling (bivalent VHH's) or by pegylation (see for example Figure 9). A short and controllable half-life is desirable for surgical procedures, for example, which require an inhibition of platelet aggregation for a limited time period. The polypeptides of the present invention also retain binding activity at a pH and temperature outside those of usual physiological ranges, which means they may be useful in situations of extreme pH and temperature which require a modulation of platelet aggregation, such as in gastric surgery, control of gastric bleeding, assays performed at room temperature etc. The polypeptides of the present invention also exhibit a prolonged stability at extremes of pH, meaning they would be suitable for delivery by oral administration. The polypeptides of the present invention may be cost-effectively produced through fermentation in convenient recombinant host organisms such as *Escherichia coli* and yeast; unlike conventional antibodies which also require expensive mammalian cell culture facilities, achievable levels of expression are high. Examples of yields of the polypeptides of the present invention are 1 to 10 mg/ml (*E. coli*) and up to 1g/l (yeast). The polypeptides of the present invention also exhibit high binding affinity for a broad range of different antigen types, and ability to bind to epitopes not recognised by conventional antibodies; for example they display long CDR-based loop structures with

the potential to penetrate into cavities and exhibit enzyme function inhibition. Furthermore, since binding often occurs through the CDR3 loop only, it is envisaged that peptides derived from CDR3 might be used therapeutically (Desmyter *et al., J Biol Chem*, 2001, 276: 26285-90). The polypeptides of the invention are also able to retain full binding capacity as fusion protein with an enzyme or toxin. Furthermore, it might be expected that the undesirable thrombocytopenia caused by Fc:Fc receptor mediated activation of platelet aggregation and/or F(ab')(2)-mediated crosslinking of platelets which has been observed when using intact IgG or F(ab')(2) therapeutically *in vivo* (see Cauwenberghs N. *et al*, Arteriosclerosis, Thrombosis and Vascular biology, 2000, 20: 1347), will be avoided in the use of VHH, since VHH contains no Fc and it is not bivalent. Thus the polypeptides represented by SEQ ID NOs: 1 to 9 homologues or functional portions thereof provide a considerable cost and time saving in the treatment and diagnosis of conditions related to platelet aggregation, and the patient in need of said polypeptides would encounter fewer of the problems associated with conventional agents.

Platelet aggregation is the process wherein vWF-bound collagen adheres to platelets and/or platelet receptors (examples of both are gpIa/IIa, gpIb, or collagen), ultimately resulting in platelet activation. Platelet activation leads to fibrinogen binding, and finally to platelet aggregation. It is within the scope of the present invention to provide polypeptides which modulate the processes which comprise platelet aggregation such as vWF-collagen binding, vWF-platelet receptor adhesion, collagen-platelet receptor adhesion, platelet activation, fibrinogen binding and/or platelet aggregation. Said polypeptides are derived from camelid antibodies directed towards vWF, vWF A1 or A3 domains, gpIb, gpIa/IIa, or collagen, and share the same advantages as the polypeptides represented by SEQ ID NOs: 1 to 9, as described above.

The aggregation-related aspect of the invention is not limited to polypeptides represented by SEQ ID NOs: 1 to 9, but may be extended to encompass polypeptides comprising camelidae antibodies of any class directed towards vWF, vWF A1 or A3 domains, gpIb, gpIa/IIa, or collagen. These polypeptides include the full length camelid antibodies, namely Fc and VHH domains. These polypeptides are known herein as "other aggregation-related polypeptides".

The present invention relates to SEQ ID NOs: 1 to 9, other aggregation-related polypeptides, a homologous sequence thereof, and/or a functional portion thereof.

Thus, the present invention relates to SEQ ID NOs: 1 to 9, 11 to 23, other anti-TNF-alpha polypeptides, other aggregation-related polypeptides, polypeptides belonging to the new class of VHHs, a homologous sequence thereof, and/or a functional portion thereof.

As used herein, an homologous sequence of the present invention may comprise additions, deletions or substitutions of one or more amino acids, which do not substantially alter the functional characteristics of the polypeptides (*e.g.* SEQ ID NOs: 1 to 9 and 11 to 23, other anti-TNF-alpha polypeptides, other aggregation-related polypeptides, polypeptides belonging to the new class of VHHs, vWF, TNF-alpha, vWF A1 or A3 domains, gpIb, gpIa/IIa, or collagen) of the invention. For SEQ ID NOs: 1 to 9 and 11 to 23, other aggregation-related polypeptides, or polypeptides belonging to the new class of VHHs, the number of amino acid deletions or substitutions is preferably up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70 amino acids.

A homologous sequence according to the present invention includes the polypeptides represented by SEQ ID NOs: 1 to 9 and 11 to 23, other anti-TNF-alpha polypeptides, other aggregation-related polypeptides, or polypeptides belonging to the new class of VHHs, modified by the addition, deletion or substitution of amino acids to form human-like heavy chain antibody or human-like single domain heavy chain antibody, which do not substantially alter the functional characteristics of the unmodified polypeptide.

A homologous sequence of the present invention may include a polypeptide represented by any of SEQ ID NOs: 1 to 9 and 11 to 23 (or other anti-TNF-alpha polypeptides, or other aggregation-related polypeptides), which has been humanized.

A homologous sequence of the present invention may include a polypeptide represented by a polypeptide of the new class of VHH disclosed herein has been humanized. The humanization of antibodies of the new class of VHHs would further reduce the possibility of unwanted immunological reaction in a human individual upon administration.

By humanized is meant mutated so that immunogenicity upon administration in human patients is minor or nonexistent. Humanizing a polypeptide, according to the present invention, comprises a step of replacing one or more of the camelidae amino acids by their human counterpart as found in the human consensus sequence, without that polypeptide losing its typical character, *i.e.* the humanization does not significantly affect

the antigen binding capacity of the resulting polypeptide. Such methods are known by the skilled addressee. A humanization technique may also be performed by a method comprising at least the replacement of the hallmark amino acid at position 45, possibly combined with a replacement of one or more of the other hallmark amino acids at position 37, 44 and 47; numbering according to the Kabat numbering.

SEQ ID NOs 1 and 13 display more than 90% amino acid sequence homology to human VH framework regions and therefore said VHH might be administered to patients directly without expectation of an immune response therefrom, and without the additional burden of humanisation. Therefore, one aspect of the present invention allows for the direct administration of the polypeptide comprising SEQ ID NOs: 1 and 13, homologous sequence thereof, and/or a functional portion thereof to a patient in need of the same.

A homologous sequence according to the present invention may include a sequence corresponding to the sequence of SEQ ID NOs: 1 to 9 and 11 to 23 (other anti-TNF-alpha polypeptides, other aggregation-related polypeptides, or polypeptides belonging to the new class of VHHs) which exists in other *Camelidae* species such as, for example, camel, dromedary, alpaca, guanaco etc.

Where homologous sequence indicates sequence identity, it means a sequence which presents a high sequence identity (more than 70%, 75%, 80%, 85%, 90%, 95% or 98% sequence identity) with SEQ ID NOs: 1 to 9 and 11 to 23, or other anti-TNF-alpha polypeptides, other aggregation-related polypeptides, or polypeptides belonging to the new class of VHHs specific for vWF, TNF-alpha, vWF A1 or A3 domains, gpIb, gpIa/IIa, or collagen, and is preferably characterised by similar properties of the parent sequence, namely affinity, said identity calculated using known methods.

Alternatively, an homologous sequence may also be any amino acid sequence resulting from allowed substitutions at any number of positions of the parent sequence according to the formula below:
Ser substituted by Ser, Thr, Gly, and Asn;
Arg substituted by one of Arg, His, Gln, Lys, and Glu;
Leu substituted by one of Leu, Ile, Phe, Tyr, Met, and Val;
Pro substituted by one of Pro, Gly, Ala, and Thr;
Thr substituted by one of Thr, Pro, Ser, Ala, Gly, His, and Gln;
Ala substituted by one of Ala, Gly, Thr, and Pro;
Val substituted by one of Val, Met, Tyr, Phe, Ile, and Leu;

Gly substituted by one of Gly, Ala, Thr, Pro, and Ser;
Ile substituted by one of Ile, Met, Tyr, Phe, Val, and Leu;
Phe substituted by one of Phe, Trp, Met, Tyr, Ile, Val, and Leu;
Tyr substituted by one of Tyr, Trp, Met, Phe, Ile, Val, and Leu;
His substituted by one of His, Glu, Lys, Gln, Thr, and Arg;
Gln substituted by one of Gln, Glu, Lys, Asn, His, Thr, and Arg;
Asn substituted by one of Asn, Glu, Asp, Gln, and Ser;
Lys substituted by one of Lys, Glu, Gln, His, and Arg;
Asp substituted by one of Asp, Glu, and Asn;
Glu substituted by one of Glu, Asp, Lys, Asn, Gln, His, and Arg;
Met substituted by one of Met, Phe, Ile, Val, Leu, and Tyr.

A homologous nucleotide sequence according to the present invention may refer to nucleotide sequences of more than 50, 100, 200, 300, 400, 500, 600, 800 or 1000 nucleotides able to hybridize to the reverse-complement of the nucleotide sequence capable of encoding SEQ ID NOs: 1 to 9 and 11 to 23, or other anti-TNF-alpha polypeptides, other aggregation-related polypeptides, or polypeptides belonging to the new class of VHHs, vWF, TNF-alpha, vWF A1 or A3 domains, gpIb, gpIa/IIa, or collagen under stringent hybridisation conditions (such as the ones described by Sambrook *et al.*, Molecular Cloning, Laboratory Manuel, Cold Spring, Harbor Laboratory press, New York).

As used herein, a functional portion refers to a sequence of SEQ ID NOs: 1 to 9 and 11 to 23, or other anti-TNF-alpha polypeptides, other aggregation-related polypeptides, or polypeptides belonging to the new class of VHHs specific for vWF, TNF-alpha, vWF A1 or A3 domains, gpIb, gpIa/IIa, or collagen that is of sufficient size such that the interaction of interest is maintained with affinity of 1×10^{-6} M or better.

Alternatively, a functional portion of any of SEQ ID NOs: 11 to 23 is a polypeptide which comprises a partial deletion of the complete amino acid sequence and which still maintains the binding site(s) and protein domain(s) necessary for the binding of and interaction with TNF-alpha.

Alternatively, according to the aggregation-related aspect of the invention, a functional portion of SEQ ID NOs:1, 2, 8 or 9 is a polypeptide which comprises a partial deletion of the complete amino acid sequence and which still maintains the binding site(s) and protein domain(s) necessary for the binding of and interaction with the A3 domain of vWF.

Alternatively, according to the aggregation-related aspect of the invention, a functional portion of any of SEQ ID NOs: 3 to 7 is a polypeptide which comprises a partial deletion of the complete amino acid sequence and which still maintains the binding site(s) and protein domain(s) necessary for the binding of and interaction with the A1 domain of vWF.

Alternatively a functional portion of other anti-TNF-alpha peptides, other aggregation-related polypeptides or polypeptides belonging to the new class of VHHs is a polypeptide which comprises a partial deletion of the complete amino acid sequence and which still maintains the binding site(s) and protein domain(s) necessary for the binding of and interaction with the antigen against which it is directed. It includes, but is not limited to the VHH domains.

As used herein, a functional portion as it refers to the polypeptide sequence of SEQ ID NOs: 1 to 9 and 11 to 23, or other anti-TNF-alpha polypeptides, other aggregation-related polypeptides, or polypeptides belonging to the new class of VHHs, TNF-alpha, vWF, vWF A1 or A3 domains, gpIb, gpIa/IIa, or collagen refers to less than 100% of the sequence (*e.g.*, 99%, 90%, 80%, 70%, 60% 50% etc.), but comprising 5 or more amino acids or 15 or more nucleotides.

A portion as it refers to the polypeptide of any of SEQ ID NOs: 1 to 9 and 11 to 23 (or other other anti-TNF-alpha polypeptides, aggregation-related polypeptides, or polypeptides belonging to the new class of VHHs), refers to less than 100% of the sequence (*e.g.*, 99%, 90%, 80%, 70%, 60% 50% etc.), but comprising 5 or more amino acids or 15 or more nucleotides.

One embodiment of the present invention is a polypeptide derived from a *Camelidae* species single domain heavy chain antibody comprising at position 103 a tryptophan residue and at position 45 an amino acid residue selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tyrosine, tryptophan, methionine, serine, threonine, asparagine, glutamine, said positions determined according to the Kabat numbering, said antibody directed to an antigen.

Another embodiment of the present invention is a polypeptide as defined above consisting of a sequence corresponding to SEQ ID NO: 1 or SEQ ID NO: 13.

Another embodiment of the present invention is a polypeptide derived from a *Camelidae* species single domain heavy chain antibody directed against a Tumor Necrosis Factor alpha (TNF-alpha) peptide antigen.

Another embodiment of the present invention is a polypeptide as defined above consisting of a sequence corresponding to any of SEQ ID NOs: 11 to 23.

Another embodiment of the present invention is a polypeptide derived from a *Camelidae* species single domain heavy chain antibody directed against a von Willebrand Factor (vWF) peptide antigen, a vWF A1 domain peptide antigen, a vWF A3 domain peptide antigen , a glycoprotein Ib peptide antigen, a glycoprotein Ia/IIa peptide antigen or a collagen peptide antigen.

Another embodiment of the present invention is a polypeptide as defined above consisting of a sequence corresponding to any of SEQ ID NOs: 1 to 9.

Another embodiment of the present invention is a polypeptide as defined above which is a humanised polypeptide.

Another embodiment of the present invention is a polypeptide comprising a polypeptide as defined above, or an homologous sequence of a polypeptide as defined above, or a portion of an homologous sequence of a polypeptide as defined above.

Another embodiment of the present invention is a nucleic acid encoding a polypeptide as defined above.

Another embodiment of the present invention is a polypeptide as defined above or a nucleic acid as defined above for use as a medicine.

Another embodiment of the present invention is a method of identifying an agent that modulates the binding of a polypeptide as defined above to said antigen comprising:

(a) contacting a polypeptide as defined above with said antigen, a homologous sequence of said antigen, and/or a functional portion of said antigen, in the presence and absence of a candidate modulator under conditions permitting binding between said polypeptide and antigen, and

(b) measuring the binding between the polypeptide and antigen of step (a), wherein a decrease in binding in the presence of said candidate modulator,

relative to the binding in the absence of said candidate modulator identified said candidate modulator as an agent that modulates the binding of a polypeptide as defined above and said antigen.

Another embodiment of the present invention is a method of identifying an agent that modulates TNF-alpha-mediated disorders through the binding of a polypeptide as defined above to TNF-alpha comprising

(a) contacting a polypeptide as defined above with said antigen, a homologous sequence of said antigen, and/or a functional portion of said antigen, in the presence and absence of a candidate modulator under conditions permitting binding between said polypeptide and antigen, and

(b) measuring the binding between the polypeptide and antigen of step (a), wherein a decrease in binding in the presence of said candidate modulator, relative to the binding in the absence of said candidate modulator identified said candidate modulator as an agent that modulates TNF-alpha-mediated disorders.

Another embodiment of the present invention is a method of identifying an agent that modulates the binding of TNF-alpha to its receptor through the binding of a polypeptide as defined above to TNF-alpha comprising

(a) contacting a polypeptide as defined above with TNF-alpha, a homologous sequence of TNF-alpha, or a functional portion of TNF-alpha, in the presence and absence of a candidate modulator under conditions permitting binding between said polypeptide and TNF-alpha, and

(b) measuring the binding between the polypeptide and TNF-alpha of step (a), wherein a decrease in binding in the presence of said candidate modulator, relative to the binding in the absence of said candidate modulator identified said candidate modulator as an agent that modulates the binding of TNF-alpha to its receptor.

Another embodiment of the present invention is a method of identifying an agent that modulates platelet aggregation through the binding of a polypeptide as defined above and said antigen comprising

(a) contacting a polypeptide as defined above with said antigen, a homologous sequence of said antigen, and/or a functional portion of said antigen, in the presence and absence of a candidate modulator under conditions permitting binding between said polypeptide and antigen, and

(b) measuring the binding between the polypeptide and antigen of step (a), wherein a decrease in binding in the presence of said candidate modulator, relative to the binding in the absence of said candidate modulator identified said candidate modulator as an agent that modulates platelet aggregation.

Another embodiment of the present invention is a kit for screening for agents that modulate polypeptide-antigen binding according to the method as defined above.

Another embodiment of the present invention is a kit for screening for agents that modulate TNF-alpha-mediated disorders according to the methods as defined above.

Another embodiment of the present invention is a kit for screening for agents that modulate platelet aggregation according to the method as defined above.

Another embodiment of the present invention is an unknown agent that modulates the binding of the polypeptides as defined above to said antigen, identifled according to the method as defined above.

Another embodiment of the present invention is an unknown agent that modulates TNF-alpha-mediated disorders, identified according to the methods as defined above.

Another embodiment of the present invention is an unknown agent that modulates platelet aggregation identified according to the method as defined above.

Another embodiment of the present invention is a use of a polypeptide as defined above, a nucleic acid as defined above, or an agent as defined above for the preparation of a medicament for treating and/or preventing and/or alleviating conditions relating to immune and inflammatory reactions.

Another embodiment of the present invention is a use of a polypeptide according to any of as defined above, a nucleic acid as defined above, or an agent as defined above for the treatment of a condition relating to immune and inflammatory reactions.

Another embodiment of the present invention is a use of a polypeptide as defined above, a nucleic acid as defined above, or an agent as defined above for the preparation of a medicament for treating and/or preventing and/or alleviating symptoms of inflammatory diseases.

Another embodiment of the present invention is a use of a polypeptide according to any of as defined above, a nucleic acid as defined above, or an agent as defined above for the preparation of a medicament for the treatment of a condition relating to immune and inflammatory reactions.

Another embodiment of the present invention is a use of a polypeptide as defined above, a nucleic acid as defined above, or an agent as defined above for the preparation of a medicament for treating and/or preventing and/or alleviating conditions relating to one or more or rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel disease and multiple sclerosis.

Another embodiment of the present invention is a use of a polypeptide as defined above, a nucleic acid as defined above or an agent as defined above for the preparation of a medicament for treating and/or preventing and/or alleviating conditions relating to platelet aggregation or dysfunction thereof.

Another embodiment of the present invention is a use of a polypeptide as defined above, a nucleic acid as defined above or an agent as defined above for the preparation of a medicament for treating and/or preventing and/or alleviating conditions arising from one or more of transient cerebral ischemic attack, unstable angina pectoris, cerebral infarction, myocardial infarction, peripheral arterial occlusive disease, restenosis, coronary by-pass graft, or coronary artery valve replacement and coronary interventions such angioplasty, stenting, or atherectomy.

Another embodiment of the present invention is a use of a polypeptide as defined above, a nucleic acid as defined above or an agent as defined above for the preparation of a medicament for the prevention of the formation of a non-occlusive thrombus.

Another embodiment of the present invention is a use of a polypeptide as defined above, a nucleic acid as defined above or an agent as defined above for the preparation of a medicament for the prevention of the formation of an occlusive thrombus.

Another embodiment of the present invention is a use of a polypeptide as defined above, a nucleic acid as defined above or an agent as defined above for the preparation of a medicament for the prevention of arterial thrombus formation.

Another embodiment of the present invention is a use of a polypeptide as defined above, a nucleic acid as defined above or an agent as defined above for the preparation of a medicament for the prevention of acute coronary occlusion.

Another embodiment of the present invention is a use of a polypeptide as defined above, a nucleic acid as defined above or an agent as defined above for the preparation of a medicament for maintaining the patency of diseased arteries.

Another embodiment of the present invention is a use of a polypeptide as defined above, a nucleic acid as defined above or an agent as defined above for the preparation of a medicament for the prevention of restenosis.

Another embodiment of the present invention is a use of a polypeptide as defined above, a nucleic acid as defined above or an agent as defined above for the preparation of a medicament for the prevention of restenosis after PCTA or stenting.

Another embodiment of the present invention is a polypeptide as defined above, a nucleic acid as defined above or an agent as defined above for the preparation of a medicament for the prevention of thrombus formation in stenosed arteries.

Another embodiment of the present invention is a use of a polypeptide as defined above, a nucleic acid as defined above or an agent as defined above for the preparation of a medicament for the prevention of hyperplasia after angioplasty, atherectomy or arterial stenting

Another embodiment of the present invention is a use of a polypeptide as defined above, a nucleic acid as defined above or an agent as defined above for the preparation of a medicament for the prevention of unstable angina.

Another embodiment of the present invention is a use of a polypeptide as defined above, a nucleic acid as defined above or an agent as defined above for the preparation of a medicament for the prevention or treatment of occlusive syndrome in a vascular system.

Another embodiment of the present invention is a method of diagnosing a disease or disorder characterised by a dysfunction of a polypeptide comprising the antigen as defined above comprising:

(a) contacting a sample with a polypeptide as defined above, and

(b) detecting binding of said polypeptide to said sample, and

(c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disease or disorder characterised by dysfunction of the antigen as defined above.

Another embodiment of the present invention is a method of diagnosing a disease or disorder characterised by the dysfunction of TNF-alpha comprising:

(a) contacting a sample with a polypeptide as defined above, and

(b) detecting binding of said polypeptide to said sample, and

(c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disease or disorder characterised by dysfunction of TNF-alpha.

Another embodiment of the present invention is a method of diagnosing a disease or disorder characterised dysfunction of platelet aggregation comprising:

(a) contacting a sample with a polypeptide as defined above, and

(b) detecting binding of said polypeptide to said sample, and

(c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disease or disorder characterised by dysfunction of platelet aggregation.

Another embodiment of the present invention is a kit for screening for a disease or disorder as defined above, using the methods as defined above.

Another embodiment of the present invention is a kit as defined above comprising an isolated polypeptide as defined above.

Another embodiment of the present invention is a use of a polypeptide as defined above for the purification of said antigen.

Another embodiment of the present invention is a use of a polypeptide as defined above for the purification TNF-alpha.

Another embodiment of the present invention is a use of a polypeptide as defined above for the purification of von Willebrand factor, von Willebrand factor A1 domain, von Willebrand factor A3 domain, gpIb, gpIa/IIa, or collagen type I.

Another embodiment of the present invention is a use of a polypeptide as defined above for inhibiting the interaction between TNF-alpha and one or more TNF-alpha receptors.

Another embodiment of the present invention is a use of a polypeptide as defined above for inhibiting the interaction between von Willebrand factor and collagen and/or platelet receptors.

Another embodiment of the present invention is a use of a polypeptide as defined above for inhibiting platelet aggregation.

Another embodiment of the present invention is a use of a polypeptide as defined above or a nucleic acid as defined above for the manufacture of a medicament to be administered orally, for the prevention or treatment of any of the disorders as defined above.

Another embodiment of the present invention is a method for producing a polypeptide as defined above comprising the steps of:

(a) obtaining double stranded DNA encoding a camelidae species single domain heavy chain antibody directed to said antigen,

(b) selecting and screening DNA comprising at position 103 a tryptophan residue and at position 45 an amino acid residue selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tyrosine, tryptophan, methionine, serine, threonine, asparagine, glutamine, and

(c) cloning and expressing the DNA selected in step (b).

Another embodiment of the present invention is a method of producing a polypeptide as defined above comprising

(a) culturing host cells comprising nucleic acid capable of encoding a polypeptide as defined above, under conditions allowing the expression of the polypeptide, and,

(b) recovering the produced polypeptide from the culture.

Another embodiment of the present invention is a method as defined above, wherein said host cells are bacterial or yeast.

Another embodiment of the present invention is a method for humanising a Camelidae antyibody or antibody fragment comprising introducing in the amino acid sequence of said antibody or antibody fragment, at position 103 a tryptophan residue and at position 45 an amino acid residue selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tyrosine, tryptophan, methionine, serine, threonine, asparagine, glutamine said positions determined according to the Kabat numbering.

Another embodiment of the present invention is a method for humanising a Camelidae antibody or antibody fragment comprising introducing in the nucleic acid sequence of said antibody or antibody fragment, at amino acid position 103 a codon encoding a tryptophan residue and at amino acid position 45 a codon encoding an amino acid residue selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tyrosine, tryptophan, methionine, serine, threonine, asparagine, glutamine said positions determined according to the Kabat numbering.

In the aggregation-related aspect of the invention, an embodiment is a polypeptide comprising the sequence of a *Camelidae* antibody directed against a von Willebrand factor peptide, a von Willebrand factor A1 domain peptide, a von Willebrand factor A3 domain peptide, a gpIb peptide, a gpIa/IIa peptide, or a collagen peptide, a homologous sequence of said antibody sequence and/or a functional portion of said antibody sequence. A functional portion might include a VHH domain of said *Camelidae* antibody.

In the aggregation-related aspect of the invention, another embodiment is a polypeptide comprising a sequence represented by SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 9, a homologous sequence thereof and/or a functional portion thereof.

In the aggregation-related aspect of the invention, another embodiment is a polypeptide as defined above wherein said polypeptide is a VHH molecule comprising said polypeptide or an Fc molecule comprising said polypeptide, or wherein said polypeptide is pegylated.

In the aggregation-related aspect of the invention, another embodiment is a polypeptide as defined above for use as a medicament.

In the aggregation-related aspect of the invention, another embodiment is a polypeptide as defined above for the preparation of a medicament for treating and/or preventing and/or alleviating conditions relating to platelet aggregation or dysfunction thereof.

In the aggregation-related aspect of the invention, another embodiment is a polypeptide as defined above for the preparation of a medicament for treating and/or preventing and/or alleviating conditions arising from transient cerebral ischemic attack, unstable angina pectoris, cerebral infarction, myocardial infarction, peripheral arterial occlusive disease, restenosis, coronary by-pass graft, or coronary artery valve replacement and coronary interventions such angioplasty, stenting, or atherectomy.

In the aggregation-related aspect of the invention, another embodiment is a method of producing a polypeptide as defined above comprising

(a) culturing host cells comprising nucleic acid capable of encoding a polypeptide as defined above, under conditions allowing the expression of the polypeptide, and,

(b) recovering the produced polypeptide from the culture.

In the aggregation-related aspect of the invention, another embodiment is a method as defined above, wherein said host cells are bacterial or yeast.

In the aggregation-related aspect of the invention, another embodiment is a method of identifying an agent that modulates platelet aggregation comprising

(a) contacting a polypeptide as defined above with a polypeptide corresponding to its target, a homologous sequence of said target, and/or a functional portion of said target, in the presence and absence of a candidate modulator under conditions permitting binding between said polypeptides, and

(b) measuring the binding between the polypeptides of step (a), wherein a decrease in binding in the presence of said candidate modulator, relative to the binding in the absence of said candidate modulator identified said candidate modulator as an agent that modulate platelet aggregation.

In the aggregation-related aspect of the invention, another embodiment is a kit for screening for agents that modulate platelet aggregation according to the method as defined above.

In the aggregation-related aspect of the invention, another embodiment is an unknown agent that modulates platelet aggregation identified according to the method as defined above.

In the aggregation-related aspect of the invention, another embodiment is a method of diagnosing a disease or disorder characterised by dysfunction of platelet aggregation comprising the steps of:

(a) contacting a sample with a polypeptide as defined above, and

(b) detecting binding of said polypeptide to said sample, and

(c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disease or disorder characterised by dysfunction of platelet aggregation.

In the aggregation-related aspect of the invention, another embodiment is a kit for screening for diagnosing a disease or disorder characterised by dysfunction of platelet aggregation according to the method as defined above.

In the aggregation-related aspect of the invention, another embodiment is a kit as defined above comprising an isolated polypeptide as defined above.

In the aggregation-related aspect of the invention, another embodiment is a use of a polypeptide as defined above for the purification of von Willebrand factor, von Willebrand factor A1 domain, von Willebrand factor A3 domain, gpIb, gpIa/IIa, or collagen.

In the aggregation-related aspect of the invention, another embodiment is a nucleic acid capable of encoding a polypeptide as defined above.

In the aggregation-related aspect of the invention, another embodiment is a nucleic acid as defined above for use as a medicament.

In the aggregation-related aspect of the invention, another embodiment is a nucleic acid as defined above for the preparation of a medicament for treating and/or preventing and/or alleviating conditions relating to platelet aggregation and /or a dysfunction of platelet aggregation.

In the aggregation-related aspect of the invention, another embodiment is a nucleic acid as defined above for the preparation of a medicament for treating and/or preventing and/or

alleviating conditions arising from coronary interventions such as angioplasty, stenting, or atherectomy.

In the aggregation-related aspect of the invention, another embodiment is a nucleic acid as defined above for the preparation of a medicament for treating and/or preventing and/or alleviating conditions arising from transient cerebral ischemic attack, unstable angina pectoris, cerebral infarction, myocardial infarction, peripheral arterial occlusive disease, restenosis , coronary by-pass graft, or coronary artery valve replacement.

In the aggregation-related aspect of the invention, another embodiment is a kit as defined above comprising an isolated nucleic acid as defined above.

In the aggregation-related aspect of the invention, another embodiment is a use of a nucleic acid as defined above for the purification of von Willebrand factor, von Willebrand factor A1 domain, von Willebrand factor A3 domain, gpIb, gpIa/IIa, or collagen.

In the aggregation-related aspect of the invention, another embodiment is a use of a polypeptide as defined above for inhibiting the interaction between von Willebrand factor and collagen and/or platelet receptors.

In the aggregation-related aspect of the invention, another embodiment is a use of a polypeptide as defined above for inhibiting platelet aggregation.

In the aggregation-related aspect of the invention, another embodiment is a use of a polypeptide according to any of as defined above, a nucleic acid as defined above, an agent as defined above for the preparation of a composition for treating conditions relating to platelet aggregation or dysfunction thereof.

In the aggregation-related aspect of the invention, another embodiment is a composition comprising a polypeptide as defined above, a nucleic acid as defined above or an agent as defined above.

In the aggregation-related aspect of the invention, another embodiment is a polypeptide as defined above for the preparation of a medicament for the prevention of the formation of a non-occlusive thrombus.

In the aggregation-related aspect of the invention, another embodiment is a polypeptide as defined above for preparation of a medicament for the prevention of the formation of an occlusive thrombus.

In the aggregation-related aspect of the invention, another embodiment is a polypeptide as defined above, for the preparation of a medicament for the prevention of arterial thrombus formation.

In the aggregation-related aspect of the invention, another embodiment is a polypeptide as defined above for the preparation of a medicament for the prevention of acute coronary occlusion.

In the aggregation-related aspect of the invention, another embodiment is a polypeptide as defined above for the preparation of a medicament for maintaining the patency of diseased arteries.

In the aggregation-related aspect of the invention, another embodiment is a polypeptide as defined above for the preparation of a medicament for the prevention of restenosis.

In the aggregation-related aspect of the invention, another embodiment is a polypeptide as defined above for the preparation of a medicament for the prevention of restenosis after PCTA or stenting.

In the aggregation-related aspect of the invention, another embodiment is a polypeptide as defined above for the preparation of a medicament for the prevention of thrombus formation in stenosed arteries.

In the aggregation-related aspect of the invention, another embodiment is a polypeptide as defined above for the preparation of a medicament for the prevention of hyperplasia after angioplasty, atherectomy or arterial stenting

In the aggregation-related aspect of the invention, another embodiment is a polypeptide as defined above for the preparation of a medicament for the prevention of unstable angina.

In the aggregation-related aspect of the invention, another embodiment is a polypeptide as defined above for the preparation of a medicament for the prevention or treatment of occlusive syndrome in a vascular system.

In the aggregation-related aspect of the invention, another embodiment is a use of a polypeptide as defined above for the manufacture of a medicament to be administered orally, for the prevention or treatment of disorders as defined above.

Screening

In one aspect of the invention, one can use a polypeptide from the new class of VHHs (e.g SEQ ID NO: 1 and 11), a homologous sequence thereof, or a functional portion thereof, which has been raised against an antigen (e.g. vWF, TNF-alpha) in order to screen for agents that modulate the binding of the polypeptide to said antigen. When identified in an assay that measures binding or said polypeptide displacement alone, agents will have to be subjected to functional testing to determine whether they would modulate the action of the antigen *in vivo*. Examples of screening assays are given below primarily in respect of SEQ ID NOs: 1 as the polypeptide and vWF as the target. However, the method below equally applies to screening for candidate modulators that bind to an antigen, using a peptide of the new class of VHHs as the polypeptide, and the antigen against which the VHH is directed in place of vWF.

Alternatively, one can use a polypeptide of the invention (*e.g.* SEQ ID NOs: 11 to 23 or other anti-TNF-alpha polypeptides, a homologous sequence thereof, or a functional portion thereof) in order to screen for agents that modulate the binding of the polypeptide to a TNF-alpha. When identified in an assay that measures binding of said polypeptide displacement alone, agents will have to be subjected to functional testing to determine whether they act as modulators of binding between TNF-alpha and TNF-alpha receptor.

In the aggregation-related aspect of the invention, one can use a polypeptide of the invention (*e.g.* SEQ ID NOs: 1 to 9 or other aggregation-related polypeptides, a homologous sequence thereof, or a functional portion thereof) in order to screen for agents that modulate the binding of the polypeptide to a vWF (or gpIb, gpIa/IIa, or collagen). When identified in an assay that measures binding or said polypeptide displacement alone, agents will have to be subjected to functional testing to determine whether they act as modulators of platelet aggregation.

In an example of a displacement experiment, phage or cells expressing vWF or a fragment thereof are incubated in binding buffer with, for example, a polypeptide represented by SEQ ID NO: 1 which has been labeled, in the presence or absence of increasing concentrations of a candidate modulator. To validate and calibrate the assay, control competition reactions using increasing concentrations of said polypeptide and which is unlabeled, can be performed. After incubation, cells are washed extensively, and bound, labeled polypeptide is measured as appropriate for the given label (*e.g.*, scintillation counting, fluorescence, etc.). A decrease of at least 10% in the amount of labeled polypeptide bound in the presence of candidate modulator indicates displacement of binding by the candidate modulator. Candidate modulators are considered to bind specifically in this or other assays described herein if they displace 50% of labeled polypeptide (sub-saturating polypeptide dose) at a concentration of 1 μM or less. Of course, the above method might easily be applied to screening for candidate modulators which alter the binding between the polypeptides represented by SEQ ID NOs: 1 to 9 or "other aggregation-related polypeptides" and macromolecules involved in platelet aggregation such as, for example, vWF, gpIb, gpIa/IIa, or collagen, or a fragment thereof. It may also be easily be applied to screening for candidate modulators which alter the binding between the polypeptides represented by SEQ ID NOs: 11 to 23 or "other anti-TNF-alpha polypeptides " and TNF-alpha or a fragment thereof.

Alternatively, binding or displacement of binding can be monitored by surface plasmon resonance (SPR). Surface plasmon resonance assays can be used as a quantitative method to measure binding between two molecules by the change in mass near an immobilized sensor caused by the binding or loss of binding of , for example, the polypeptide represented by SEQ ID NO: 1 from the aqueous phase to a vWF, or fragment thereof immobilized in a membrane on the sensor. This change in mass is measured as resonance units versus time after injection or removal of the said polypeptide or candidate modulator and is measured using a Biacore Biosensor (Biacore AB). vWF, or fragment thereof can be for example immobilized on a sensor chip (for example, research grade CM5 chip; Biacore AB) in a thin film lipid membrane according to methods described by Salamon et al. (Salamon *et al.*, 1996, Biophys J. 71: 283-294; Salamon *et al.*, 2001, Biophys. J. 80: 1557-1567; Salamon *et al.*, 1999, Trends Biochem. Sci. 24: 213-219, each of which is incorporated herein by reference.). Sarrio *et al.* demonstrated that SPR can be used to detect ligand binding to the GPCR A(1) adenosine receptor immobilized in a lipid layer on the chip (Sarrio *et al.*, 2000, Mol. Cell. Biol. 20: 5164-5174, incorporated herein by reference). Conditions for the binding of a polypeptide such as, for example, a polypeptide represented by SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8 or 9 to a vWF, or fragment

thereof in an SPR assay can be fine-tuned by one of skill in the art using the conditions reported by Sarrio *et al.* as a starting point. Of course, the above method might easily be applied to screening for candidate modulators which alter the binding between " other aggregation-related polypeptides " and macromolecules involved in platelet aggregation such as, for example, vWF, gpIb, gpIa/IIa, or collagen, or a fragment thereof. It may also be easily be applied to screening for candidate modulators which alter the binding between the polypeptides represented by SEQ ID NOs: 11 to 23 or "other anti-TNF-alpha polypeptides " and TNF-alpha or a fragment thereof.

SPR can assay for modulators of binding in at least two ways. First, a polypeptide represented by SEQ ID NO: 1, for example, can be pre-bound to immobilized vWF, or fragment thereof, followed by injection of candidate modulator at a concentration ranging from 0.1 nM to 1 µM. Displacement of the bound polypeptide can be quantitated, permitting detection of modulator binding. Alternatively, the membrane-bound vWF, or fragment thereof can be pre-incubated with a candidate modulator and challenged with, for example, a polypeptide represented by SEQ ID NO: 1. A difference in binding affinity between said polypeptide and vWF, or fragment thereof pre-incubated with the modulator, compared with that between said polypeptide and vWF, or fragment thereof in absence of the modulator will demonstrate binding or displacement of said polypeptide in the presence of modulator. In either assay, a decrease of 10% or more in the amount of said polypeptide bound in the presence of candidate modulator, relative to the amount of said polypeptide bound in the absence of candidate modulator indicates that the candidate modulator inhibits the interaction of vWF, or fragment thereof and said polypeptide. Of course, the above method might easily be applied to screening for candidate modulators which alter the binding between the polypeptides represented by SEQ ID NOs: 2 to 9 or " other aggregation-related polypeptides " and macromolecules involved in platelet aggregation such as, for example, vWF, gpIb, gpIa/IIa, or collagen, or a fragment thereof. It may also be easily be applied to screening for candidate modulators which alter the binding between the polypeptides represented by SEQ ID NOs: 11 to 23 or "other anti-TNF-alpha polypeptides " and TNF-alpha or a fragment thereof.

Another method of detecting inhibition of binding of, for example, a polypeptide represented by SEQ ID NOs: 1 to 9, to vWF, or fragment thereof uses fluorescence resonance energy transfer (FRET). FRET is a quantum mechanical phenomenon that occurs between a fluorescence donor (D) and a fluorescence acceptor (A) in close proximity to each other (usually < 100 Å of separation) if the emission spectrum of D overlaps with the excitation spectrum of A. The molecules to be tested, *e.g.* a polypeptide

represented by SEQ ID NO: 1 and a vWF, or fragment thereof, are labelled with a complementary pair of donor and acceptor fluorophores. While bound closely together by the vWF: polypeptide interaction, the fluorescence emitted upon excitation of the donor fluorophore will have a different wavelength from that emitted in response to that excitation wavelength when the said polypeptide and vWF, or fragment thereof are not bound, providing for quantitation of bound versus unbound molecules by measurement of emission intensity at each wavelength. Donor fluorophores with which to label the vWF, or fragment thereof are well known in the art. Of particular interest are variants of the A. Victoria GFP known as Cyan FP (CFP, Donor (D)) and Yellow FP (YFP, Acceptor (A)). As an example, the YFP variant can be made as a fusion protein with vWF, or fragment thereof. Vectors for the expression of GFP variants as fusions (Clontech) as well as flurophore-labeled reagents (Molecular Probes) are known in the art. The addition of a candidate modulator to the mixture of fluorescently-labelled polypeptide and YFP-vWF will result in an inhibition of energy transfer evidenced by, for example, a decrease in YFP fluorescence relative to a sample without the candidate modulator. In an assay using FRET for the detection of vWF : polypeptide interaction, a 10% or greater decrease in the intensity of fluorescent emission at the acceptor wavelength in samples containing a candidate modulator, relative to samples without the candidate modulator, indicates that the candidate modulator inhibits the vWF:polypeptide interaction. Of course, the above method might easily be applied to screening for candidate modulators which alter the binding between the polypeptides represented by SEQ ID NOs: 2 to 9 or " other aggregation-related polypeptides " and macromolecules involved in platelet aggregation such as, for example, vWF, gpIb, gpIa/IIa, or collagen, or a fragment thereof. It may also be easily be applied to screening for candidate modulators which alter the binding between the polypeptides represented by SEQ ID NOs: 11 to 23 or "other anti-TNF-alpha polypeptides " and TNF-alpha or a fragment thereof.

A variation on FRET uses fluorescence quenching to monitor molecular interactions. One molecule in the interacting pair can be labelled with a fluorophore, and the other with a molecule that quenches the fluorescence of the fluorophore when brought into close apposition with it. A change in fluorescence upon excitation is indicative of a change in the association of the molecules tagged with the fluorophore:quencher pair. Generally, an increase in fluorescence of the labelled vWF, or fragment thereof is indicative that the polypeptide molecule (*e.g.* a polypeptide represented by SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8 or 9) bearing the quencher has been displaced. For quenching assays, a 10% or greater increase in the intensity of fluorescent emission in samples containing a candidate modulator, relative to samples without the candidate modulator, indicates that the

candidate modulator inhibits vWF : polypeptide interaction. Of course, the above method might easily be applied to screening for candidate modulators which alter the binding between " other aggregation-related polypeptides " and macromolecules involved in platelet aggregation such as, for example, vWF, gpIb, gpIa/IIa, or collagen, or a fragment thereof. It may also be easily be applied to screening for candidate modulators which alter the binding between the polypeptides represented by SEQ ID NOs: 11 to 23 or "other anti-TNF-alpha polypeptides " and TNF-alpha or a fragment thereof.

In addition to the surface plasmon resonance and FRET methods, fluorescence polarization measurement is useful to quantitate binding. The fluorescence polarization value for a fluorescently-tagged molecule depends on the rotational correlation time or tumbling rate. Complexes, such as those formed by vWF, or fragment thereof associating with a fluorescently labelled polypeptide (*e.g.* a fluorescently-labelled polypeptide represented by SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8 or 9), have higher polarization values than uncomplexed, labelled polypeptide. The inclusion of a candidate inhibitor of the vWF:polypeptide interaction results in a decrease in fluorescence polarization, relative to a mixture without the candidate inhibitor, if the candidate inhibitor disrupts or inhibits the interaction of vWF, or fragment thereof with said polypeptide. Fluorescence polarization is well suited for the identification of small molecules that disrupt the formation of vWF: polypeptide complexes. A decrease of 10% or more in fluorescence polarization in samples containing a candidate modulator, relative to fluorescence polarization in a sample lacking the candidate modulator, indicates that the candidate modulator inhibits the vWF: polypeptide interaction. Of course, the above method might easily be applied to screening for candidate modulators which alter the binding between " other aggregation-related polypeptides " and macromolecules involved in platelet aggregation such as, for example, vWF, gpIb, gpIa/IIa, or collagen, or a fragment thereof. It may also be easily be applied to screening for candidate modulators which alter the binding between the polypeptides represented by SEQ ID NOs: 11 to 23 or "other anti-TNF-alpha polypeptides " and TNF-alpha or a fragment thereof.

Another alternative for monitoring vWF : polypeptide interactions uses a biosensor assay. ICS biosensors have been described in the art (Australian Membrane Biotechnology Research Institute; Cornell B, Braach-Maksvytis V, King L, Osman P, Raguse B, Wieczorek L, and Pace R. "A biosensor that uses ion-channel switches" Nature 1997, 387, 580). In this technology, the association of vWF, or fragment thereof and a polypeptide (e.g. a polypeptide represented by SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8 or 9) is coupled to the closing of gramacidin-facilitated ion channels in suspended membrane

bilayers and thus to a measurable change in the admittance (similar to impedence) of the biosensor. This approach is linear over six orders of magnitude of admittance change and is ideally suited for large scale, high throughput screening of small molecule combinatorial libraries. A 10% or greater change (increase or decrease) in admittance in a sample containing a candidate modulator, relative to the admittance of a sample lacking the candidate modulator, indicates that the candidate modulator inhibits the interaction of vWF, or fragment thereof and said polypeptide. It is important to note that in assays testing the interaction of vWF, or fragment thereof with a polypeptide (such as for example, a polypeptide represented by SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8 or 9), it is possible that a modulator of the interaction need not necessarily interact directly with the domain(s) of the proteins that physically interact with said polypeptide. It is also possible that a modulator will interact at a location removed from the site of interaction and cause, for example, a conformational change in the vWF. Modulators (inhibitors or agonists) that act in this manner are nonetheless of interest as agents to modulate platelet aggregation. Of course, the above method might easily be applied to screening for candidate modulators which alter the binding between "other aggregation-related polypeptides " and macromolecules involved in platelet aggregation such as, for example, vWF, gpIb, gpIa/IIa, or collagen, or a fragment thereof. It may also be easily be applied to screening for candidate modulators which alter the binding between the polypeptides represented by SEQ ID NOs: 11 to 23 or "other anti-TNF-alpha polypeptides " and TNF-alpha or a fragment thereof.

Any of the binding assays described can be used to determine the presence of an agent in a sample, e.g., a tissue sample, that binds to vWF, or fragment thereof, or that affects the binding of, for example, a polypeptide represented by SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 9 to the vWF, or fragment thereof. To do so a vWF, or fragment thereof is reacted with said polypeptide in the presence or absence of the sample, and polypeptide binding is measured as appropriate for the binding assay being used. A decrease of 10% or more in the binding of said polypeptide indicates that the sample contains an agent that modulates the binding of said polypeptide to the vWF, or fragment thereof. Of course, the above-generalized method might easily be applied to screening for candidate modulators which alter the binding between "other aggregation-related polypeptides" and macromolecules involved in platelet aggregation such as, for example, vWF, gpIb, gpIa/IIa, or collagen, or a fragment thereof. It may also be easily be applied to screening for candidate modulators which alter the binding between the polypeptides represented by SEQ ID NOs: 11 to 23 or "other anti-TNF-alpha polypeptides " and TNF-alpha or a fragment thereof.

Cells

A cell that is useful according to the invention is preferably selected from the group consisting of bacterial cells such as, for example, *E. coli*, yeast cells such as, for example, *S. cerevisiae*, *P. pastoris*, insect cells or mammal cells.

A cell that is useful according to the invention can be any cell into which a nucleic acid sequence encoding a polypeptide comprising SEQ ID NOs: 1 to 9, 11 to 23 (or other anti-TNF-alpha polypeptides, other aggregation-related polypeptides, or other polypeptides belonging to the new class of VHHs), a homologous sequence thereof and/or a functional portion thereof according to the invention can be introduced such that the polypeptide is expressed at natural levels or above natural levels, as defined herein. Preferably a polypeptide of the invention that is expressed in a cell exhibits normal or near normal pharmacology, as defined herein. Most preferably a polypeptide of the invention that is expressed in a cell comprises the nucleotide sequence capable of encoding any one of the amino acid sequences presented in Table 1 and 2 or capable of encoding an amino acid sequence that is at least 70% identical to the amino acid sequence presented in Table 1 and 2.

According to a preferred embodiment of the present invention, a cell is selected from the group consisting of COS7-cells, a CHO cell, a LM (TK-) cell, a NIH-3T3 cell, HEK-293 cell, K-562 cell or a 1321N1 astrocytoma cell but also other transfectable cell lines.

Medical treatment

In general, "therapeutically effective amount", "therapeutically effective dose" and "effective amount" means the amount needed to achieve the desired result or results (treating or preventing platelet aggregation). One of ordinary skill in the art will recognize that the potency and, therefore, an "effective amount" can vary for the various compounds that inhibit platelet aggregation used in the invention. One skilled in the art can readily assess the potency of the compound.

As used herein, the term "compound" refers to a polypeptide represented by SEQ ID NOs: 1 to 9, 11 to 23, other anti-TNF-alpha polypeptides, other aggregation-related polypeptides, or other polypeptides belonging to the new class of VHHs, a homologous sequence thereof, or a homologue thereof, or a nucleic acid capable of encoding said polypeptide or an agent identified according to the screening method described herein or said polypeptide comprising one or more derivatised amino acids.

By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, *i.e.*, the material may be administered to an individual along with the compound without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

Polypeptides of the new class of VHH's as disclosed herein is useful for treating or preventing conditions in a subject and comprises administering a pharmaceutically effective amount of a compound or composition.

Polypeptides of corresponding to SEQ ID NOs: 11 to 23 and other anti-TNF polypeptides as disclosed herein is useful for treating or preventing conditions relating to rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis in a subject and comprises administering a pharmaceutically effective amount of a compound or composition that binds TNF-alpha.

The aggregation-related aspect of the invention as disclosed herein is useful for treating or preventing a condition of platelet aggregation, in a subject and comprising administering a pharmaceutically effective amount of a compound or composition that inhibits BTK and that inhibits platelet aggregation.

Polypeptides of the new class of VHH's as disclosed herein is useful for treating or preventing conditions in a subject and comprises administering a pharmaceutically effective amount of a compound combination with another, such as, for example, aspirin.

Polypeptides of corresponding to SEQ ID NOs: 11 to 23 and other anti-TNF polypeptides as disclosed herein is useful for treating or preventing conditions relating to rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis in a subject and comprises administering a pharmaceutically effective amount of a compound combination with another, such as, for example, aspirin.

The aggregation-related aspect of the present invention relates to the use of compounds of the invention for treating or preventing a condition of platelet aggregation, in a subject and comprising administering a pharmaceutically effective amount of a compound in combination with another, such as, for example, aspirin.

The aggregation-related aspect of the present invention also relates to a bivalent construct comprising one "other polypeptide" directed to collagen and one "other polypeptide" directed to platelets (for example, gpIb and gpIa/IIa), which may be used to treat patients suffering from vWF disease. vWF disease occurs in patients suffering from a lack of or insufficient vWF.

The present invention is not limited to the administration of formulations comprising a single compound of the invention. It is within the scope of the invention to provide combination treatments wherein a formulation is administered to a patient in need thereof that comprises more than one compound of the invention.

Conditions mediated by TNF-alpha include, but are not limited to rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis.

Conditions of platelet aggregation include, but are not limited to, unstable angina, angina pectoris, embolus formation, deep vain thrombosis, hemolytic uremic syndrome, hrmolytic anemia, acute renal failure, thrombolytic complications, thrombotic thrombocytopenic purpura, disseminated intravascular comgelopathy, thrombosis, coronary heart disease, thromboembolic complications, myocardial infarction, restenosis, and atrial thrombosis formation in atrial fibrillation, chronic unstable angina, transient ischemic attacks and strokes, peripheral vascular disease, arterial thrombosis, pre-eclampsia, embolism, restenosis and/or thrombosis following angioplasty, carotid endarterectomy, anastomosis of vascular grafts, and chronic exposure to cardiovascular devices. Such conditions may also result from thromboembolism and reocculsion during and after thrombolytic therapy, after angioplasty, and after coronary artery bypass.

It is well known in the art how to determine the inhibition of platelet aggregation using the standard tests described herein, or using other similar tests. Preferably, the method would result in at least a 10% reduction in platelet aggregation, including, for example, 15%, 20%, 25%, 30%, 40%, 50%,60%, 70%, 80%, 90%, 100%, or any amount in between, more preferably by 90%.

Similarly, the method would result in at least a 10% reduction in intracellular calcium mobilisation including, for example, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%. Similarly, the method would result in at least a 10% reduction in the level of phosphorylated PLCg 2 including, for example, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%.

The reduction can be measured, for example, by comparing the optical impedence in a chronology platelet aggregometer. Any other known measurement method may also be used. For example, (1) upon collagen stimulation, the level of collagen-induced intracellular calcium mobilization increases over time and so the measurement may include measuring the level of collagen-induced intracellular calcium or (2) upon collagen stimulation, the level of phosphorylated PLCg 2 increases over time and so the measurement may include measuring the level of phosphorylated PLCg 2.

The cells can be contacted *in vitro*, for example, by adding a compound of the invention to the culture medium (by continuous infusion, by bolus delivery, or by changing the medium to a medium that contains the compound) or by adding the compound to the extracellular fluid *in vivo* (by local delivery, systemic delivery, inhalation, intravenous injection, bolus delivery, or continuous infusion). The duration of "contact" with a cell or population of cells is determined by the time the compound is present at physiologically effective levels or at presumed physiologically effective levels in the medium or extracellular fluid bathing the cell or cells. Preferably, the duration of contact is 1-96 hours, and more preferably, for 24 hours, but such time would vary based on the half-life of the compound and could be optimized by one skilled in the art using routine experimentation.

The compound useful in the present invention can be formulated as pharmaceutical compositions and administered to a mammalian host, such as a human patient or a domestic animal in a variety of forms adapted to the chosen route of administration, *i.e.*, orally or parenterally, by intranassally by inhalation, intravenous, intramuscular, topical or subcutaneous routes.

The compound of the present invention can also be administered using gene therapy methods of delivery. See, *e.g.*, U.S. Patent No. 5,399,346, which is incorporated by reference in its entirety. Using a gene therapy method of delivery, primary cells transfected with the gene for the compound of the present invention can additionally be transfected with tissue specific promoters to target specific organs, tissue, grafts, tumors, or cells.

Thus, the present compound may be systemically administered, *e.g.*, orally, in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. They may be enclosed in hard or soft shell gelatin capsules, may be compressed into tablets, or may be incorporated directly with the food of the

patient's diet. For oral therapeutic administration, the active compound may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of active compound in such therapeutically useful compositions is such that an effective dosage level will be obtained.

The tablets, troches, pills, capsules, and the like may also contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other
materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the active compound, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and devices.

The active compound may also be administered intravenously or intraperitoneally by infusion or injection. Solutions of the active compound or its salts can be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the active ingredient which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage

form must be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

For topical administration, the present compound may be applied in pure form, i.e., when they are liquids. However, it will generally be desirable to administer them to the skin as compositions or formulations, in combination with a dermatologically acceptable carrier, which may be a solid or a liquid.

Useful solid carriers include finely divided solids such as talc, clay, microcrystalline cellulose, silica, alumina and the like. Useful liquid carriers include water, hydroxyalkyls or glycols or water-alcohol/glycol blends, in which the present compound can be dissolved or dispersed at effective levels, optionally with the aid of non-toxic surfactants. Adjuvants such as fragrances and additional antimicrobial agents can be added to optimize the properties for a given use. The resultant liquid compositions can be applied from absorbent pads, used to impregnate bandages and other dressings, or sprayed onto the affected area using pump-type or aerosol sprayers.

Thickeners such as synthetic polymers, fatty acids, fatty acid salts and esters, fatty alcohols, modified celluloses or modified mineral materials can also be employed with liquid carriers to form spreadable pastes, gels, ointments, soaps, and the like, for application directly to the skin of the user.

Examples of useful dermatological compositions which can be used to deliver the compound to the skin are known to the art; for example, see Jacquet et al. (U.S. Pat. No. 4,608,392), Geria (U.S. Pat. No. 4,992,478), Smith et al. (U.S. Pat. No. 4,559,157) and Wortzman (U.S. Pat. No. 4,820,508).

Useful dosages of the compound can be determined by comparing their *in vitro* activity, and *in vivo* activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are known to the art; for example, see U.S. Pat. No. 4,938,949.

Generally, the concentration of the compound(s) in a liquid composition, such as a lotion, will be from about 0.1-25 wt-%, preferably from about 0.5-10 wt-%. The concentration in a semi-solid or solid composition such as a gel or a powder will be about 0.1-5 wt-%, preferably about 0.5-2.5 wt-%.

The amount of the compound, or an active salt or derivative thereof, required for use in treatment will vary not only with the particular salt selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or clinician. Also the dosage of the compound varies depending on the target cell, tumor, tissue, graft, or organ.

In general, however, a suitable dose will be in the range of from about 0.5 to about 5000 µg/kg of body weight per day. Upon oral administration, more might be needed e.g., from about 10 to about 1000 µg/kg of body weight per day.

Ideally, the active ingredient should be administered to achieve peak plasma concentrations of the active compound of from about 0.0005 to about 300 μM, preferably, about .001 to 100 μM, more preferably, about 0.01 to about 10 μM. This may be achieved, for example, by the intravenous injection of a concentration of the active ingredient, optionally in saline, or orally administered as a bolus.

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, *e.g.*, into a number of discrete loosely spaced administrations; such as multiple inhalations from an insufflator or by application of a plurality of drops into the eye.

An administration regimen could include long-term, daily treatment. By "long-term" is meant at least two weeks and preferably, several weeks, months, or years of duration. Necessary modifications in this dosage range may be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein. See Remington's Pharmaceutical Sciences (Martin, E.W., ed. 4), Mack Publishing Co., Easton, PA. The dosage can also be adjusted by the individual physician in the event of any complication.

Candidate modulators

The invention provides for an agent that is a modulator of TNF-alpha / TNF-alpha-receptor interactions.

The invention further provides for an agent that is a modulator of platelet aggregation.

The candidate agent may be a synthetic agent, or a mixture of agents, or may be a natural product (*e.g.* a plant extract or culture supernatant). A candidate agent according to the invention includes a small molecule that can be synthesized, a natural extract, peptides, proteins, carbohydrates, lipids etc.

Candidate modulator agents from large libraries of synthetic or natural agents can be screened. Numerous means are currently used for random and directed synthesis of saccharide, peptide, and nucleic acid based agents. Synthetic agent libraries are commercially available from a number of companies including Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich (Milwaukee, WI). Combinatorial libraries are available and can be prepared. Alternatively, libraries of natural agents in the form of bacterial, fungal, plant and animal extracts are available from e.g., Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible by methods well known in the art. Additionally, natural and synthetically produced libraries and agents are readily modified through conventional chemical, physical, and biochemical means.

Useful agents may be found within numerous chemical classes. Useful agents may be organic agents, or small organic agents. Small organic agents have a molecular weight of more than 50 yet less than about 2,500 daltons, preferably less than about 750, more preferably less than about 350 daltons. Exemplary classes include heterocycles, peptides, saccharides, steroids, and the like. The agents may be modified to enhance efficacy, stability, pharmaceutical compatibility, and the like. Structural identification of an agent may be used to identify, generate, or screen additional agents. For example, where peptide agents are identified, they may be modified in a variety of ways to enhance their stability, such as using an unnatural amino acid, such as a D-amino acid, particularly D-alanine, by functionalizing the amino or carboxylic terminus, e.g. for the amino group, acylation or alkylation, and for the carboxyl group, esterification or amidification, or the like.

For primary screening, a useful concentration of a candidate agent according to the invention is from about 10 mM to about 100 µM or more (i.e. 1 mM, 10 mM, 100 mM, 1 M etc.). The primary screening concentration will be used as an upper limit, along with nine additional concentrations, wherein the additional concentrations are determined by reducing the primary screening concentration at half-log intervals (e.g. for 9 more concentrations) for secondary screens or for generating concentration curves.

High throughput screening kit

A high throughput screening kit according to the invention comprises all the necessary means and media for performing the detection of an agent that modulates TNF-alpha/TNF-alpha receptor interactions by interacting with TNF-alpha, or fragment thereof in the presence of a polypeptide (for example, a polypeptide represented by SEQ ID NOs: 11 to 23 or other anti-TNF-alpha polypeptides), preferably at a concentration in the range of 1µM to 1 mM.

A high throughput screening kit according to the invention comprises all the necessary means and media for performing the detection of an agent that modulates platelet aggregation by interacting with a macromolecule, such as for example vWF, or fragment thereof in the presence of a polypeptide (for example, a polypeptide represented by SEQ ID NOs: 1 to 9 or other aggregation-related polypeptides), preferably at a concentration in the range of 1µM to 1 mM.

Alternatively, a high throughput screening kit according to the invention comprises all the necessary means and media for performing the detection of an agent that modulates the binding between a polypeptide of the new class of VHH (for example, a polypeptide represented by SEQ ID NOs: 1 and 13), and the antigen directed thereto (e.g. vWF in the case of SEQ ID NO:1 and TNF-alpha in the case of SEQ ID NO: 13), preferably at a concentration in the range of 1µM to 1 mM.

The kit comprises the following. Recombinant cells of the invention, comprising and expressing the nucleotide sequence encoding vWF, or fragment thereof, which are grown according to the kit on a solid support, such as a microtiter plate, more preferably a 96 well microtiter plate, according to methods well known to the person skilled in the art especially as described in WO 00/02045. Alternatively vWF, or fragment thereof is supplied in a purified form to be immobilized on, for example, a 96 well microtiter plate by the person skilled in the art. Alternatively vWF, or fragment thereof is supplied in the kit pre-immobilized on, for example, a 96 well microtiter plate. Alternatively, in cases where the macromolecule to be screened against is gpIb, gpIa/IIa, or collagen, the above embodiments would carry gpIb, gpIa/IIa, or collagen polypeptide or polynucleic acid respectively in place of vWF. Kit may contain more than one macromolcule (e.g. vWF, gpIb, gpIa/IIa, or collagen macromolecule and/or polynucleic acid). Alternatively, in cases where the macromolecule to be screened against is TNF-alpha, the above embodiments would carry TNF-alpha polypeptide or polynucleic acid respectively in place of vWF. Alternatively, in cases where the macromolecule to be screened against is an antigen (e.g. vWF or TNF-alpha, which may not necessarily be macromolecular) against which a VHH of the new class is directed, the above embodiments would carry said antigen in place of vWF. Kit may contain more than one macromolcule (e.g. vWF, TNF-alpha, gpIb, gpIa/IIa, or collagen macromolecule and/or polynucleic acid). Modulator agents according to the invention, at concentrations from about 1 µM to 1 mM or more, are added to defined wells in the presence of an appropriate concentration of polypeptide (such as for example, a polypeptide represented by any of the SEQ ID NOs: 1 to 9 or 11 to 23 or other anti-TNF-alpha polypeptides, other aggregation-related polypeptides, polypeptides belonging to the new class of VHHs) said concentration of said polypeptide preferably in the range of 1 µM to 1 mM. Kits may contain more than one polypeptide.

Binding assays are performed as according to the methods already disclosed herein and the results are compared to the baseline level of, for example vWF, or fragment thereof binding to a polypeptide, such as, for example, a polypeptide represented by SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8 or 9 or other aggregation-related polypeptide, but in the absence

of added modulator agent. Wells showing at least 2 fold, preferably 5 fold, more preferably 10 fold and most preferably a 100 fold or more increase or decrease in vWF-polypeptide binding (for example) as compared to the level of activity in the absence of modulator, are selected for further analysis.

Other Kits Useful According to the Invention

The invention provides for kits useful for screening for modulators of TNF-alpha/TNF-alpha receptor binding, as well as kits useful for diagnosis of diseases or disorders characterised by disfunction of TNF-alpha. The invention also provides for kits useful for screening for modulators of platelet aggregation, as well as kits useful for diagnosis of diseases or disorders characterised by dysregulation of platelet aggregation. The invention also provides for kits useful for screening for modulators of other diseases as well as kits for their diagnosis, said diseases characterised by one or more antigen against which the new class of VHHs disclosed herein is directed to. Kits useful according to the invention can include an isolated vWF, or fragment thereof. Alternatively, or in addition, a kit can comprise cells transformed to express vWF, or fragment thereof. In a further embodiment, a kit according to the invention can comprise a polynucleotide encoding vWF, or fragment thereof. In a still further embodiment, a kit according to the invention may comprise the specific primers useful for amplification of vWF, or fragment thereof. Alternatively, in cases where the macromolecule to be screened against is gpIb, gpIa/IIa, or collagen, the above embodiments would carry gpIb, gpIa/IIa, or collagen polypeptide or polynucleic acid, or fragment thereof respectively in place of vWF. Alternatively, in cases where the macromolecule to be screened against is TNF-alpha, the above embodiments would carry TNF-alpha or polynucleic acid, or fragment thereof respectively in place of vWF. Alternatively, in cases where the macromolecule to be screened against is an antigen (e.g. vWF or TNF-alpha, which may not necessarily be macromolecular) against which a VHH of the new class is directed, the above embodiments would carry said antigen in place of vWF. Kit may contain more than one macromolcule (e.g. vWF, TNF-alpha, gpIb, gpIa/IIa, or collagen macromolecule, antigen or polynucleic acid, or fragment thereof). Kits useful according to the invention can comprise an isolated polypeptide represented by any of the SEQ ID NOs: 1 to 9 or 11 to 23, other anti-TNF-alpha polypeptides, or other aggregation-related polypeptides, polypeptides belonging to the new class of VHHs, a homologue thereof, or a functional portion thereof. A kit according to the invention can comprise cells transformed to express said polypeptide. Kits may contain more than one polypeptide. In a further embodiment, a kit according to the invention can comprise a polynucleotide encoding a macromolecule, for example, vWF, TNF-alpha, gpIb, gpIa/IIa, or collagen, or fragment thereof. In a still further embodiment, a

kit according to the invention may comprise the specific primers useful for amplification of a macromolecule such as, for example, vWF, TNF-alpha, gpIb, gpIa/IIa, or collagen, or fragment thereof. All kits according to the invention will comprise the stated items or combinations of items and packaging materials therefore. Kits will also include instructions for use.

Medical devices

The aggregation-related aspect of the present invention also provides for invasive medical devices coated with SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8 or 9, or other polypeptide, a homologue thereof, and/or a functional portion thereof or an agent resulting from a screening method of the invention for use in devices requiring the same. Non-limiting examples of devices include surgical tubing, occlusion devices, prosthetic devices. Application for said devices include surgical procedures which require a modulation of platelet aggregation around the site of invasion.

Delivery

The present invention also relates to VHH molecules derived from antibodies raised in Camelidae species, for example in camel, dromedary, alpaca and guanaco and their use in the delivery of protein therapeutic molecules. As pointed out above, VHH molecules are about 10x smaller than IgG molecules. They are single polypeptides and very stable, resisting extreme pH and temperature conditions. Moreover, they are resistant to the action of proteases which is not the case for conventional antibodies.

One embodiment of the present invention is a VHH for use in treating, preventing and/or alleviating the symptoms of diseases requiring the delivery of a therapeutic compound that is able pass through the gastric environment without being inactivated. As known by persons skilled in the art, once in possession of said VHHs, formulation technology may be applied to release a maximum amount of VHHs in the right location (in the stomach, in the colon, etc.). This method of delivery is important for treating, prevent and/or alleviate the symptoms of disease whose targets that are located in the gut system. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of a disease requiring the delivery of a therapeutic compound that is able pass through the gastric environment without being inactivated, by orally administering to a subject a VHH specific for an antigen related to the disease.

Another embodiment of the present invention is a VHH for use in treating, preventing and/or alleviating the symptoms of diseases requiring the delivery of a therapeutic

compound to the vaginal and/or rectal tract. In a non-limiting example, a formulation according to the invention comprises VHH molecules directed against one or more targets in the form of a gel, cream, suppository, film, or in the form of a sponge or as a vaginal ring that slowly releases the active ingredient over time. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of diseases requiring the delivery of a therapeutic compound to the vaginal and/or rectal tract, by vaginally and/or rectally administering to a subject a VHH specific for an antigen related to the disease.

Another embodiment of the present invention is a VHH for use in treating, preventing and/or alleviating the symptoms of diseases requiring the delivery of a therapeutic compound to the upper respiratory tract and lung. In a non-limiting example, a formulation according to the invention, comprises VHH molecules directed against one or more targets in the form of a nasal spray (e.g. an aerosol). Since VHH molecules are small, it is they can reach their target much more effectively than therapeutic IgG molecules. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of diseases requiring the delivery of a therapeutic compound to the upper respiratory tract and lung, by administering to a subject said a VHH specific for an antigen related to the disease by inhalation.

One embodiment of the present invention is a VHH for use in treating, preventing and/or alleviating the symptoms of diseases wherein the permeability of the intestinal mucosa is increased. Because of their small size, VHH can pass through the intestinal mucosa and reach the bloodstream more efficiently in subjects suffering from diseases which cause an increase in the permeability of the intestinal mucosa, for example Crohn's disease. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of diseases wherein the permeability of the intestinal mucosa is increased, by orally administering to a subject a VHH specific for an antigen related to the disease.

This process can be even further enhanced by an additional aspect of the present invention - the use of active transport carriers. In this aspect of the invention, VHH is fused to a carrier that enhances the transfer through the intestinal wall into the bloodstream. In a non-limiting example, this "carrier" is a second VHH which is fused to the therapeutic VHH. Such fusion constructs made using methods known in the art. The "carrier" VHH binds specifically to a receptor on the intestinal wall which induces an active transfer through the wall.

One embodiment of the present invention is a VHH for use in treating, preventing and/or alleviating the symptoms of diseases requiring the delivery of a therapeutic compound that is able pass through the tissues beneath the tongue effectively. A formulation of said VHH, for example, a tablet, spray, drop is placed under the tongue and adsorbed through the mucus membranes into the capilliary network under the tongue. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of diseases requiring the delivery of a therapeutic compound that is able pass through the tissues beneath the tongue effectively, by sublingually administering to a subject a VHH specific for an antigen related to the disease.

One embodiment of the present invention is a VHH for use in treating, preventing and/or alleviating the symptoms of diseases requiring the delivery of a therapeutic compound that is able pass through the skin effectively. A formulation of said VHH, for example, a cream, film, spray, drop, patch, is placed on the skin and passes through. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of diseases requiring the delivery of a therapeutic compound that is able pass through the skin effectively, by topically administering to a subject a VHH specific for an antigen related to the disease.

In another embodiment of the present invention, a carrier VHH is used as active transport carriers for transport of a therapeutic VHH from the lung lumen to the blood. The carrier VHH binds specifically to a receptor present on the mucosal surface (bronchial epithelial cells) resulting in the active transport of the therapeutic VHH specific for bloodstream targets from the lung lumen to the blood. The carrier VHH is fused to the therapeutic VHH. Such fusion constructs made using methods known in the art. The "carrier" VHH binds specifically to a receptor on the mucosal surface which induces an active transfer through the surface.

A non-limiting example of a therapeutic target against which the VHHs of the invention may be used is TNF-alpha, which is involved in inflammatory processes. The blocking of TNF-alpha action can have an anti-inflammatory effect, which is highly desirable in certain disease states such as, for example, Crohn's disease. Current therapy consists of intravenous administration of anti-TNF-alpha antibodies. Our Examples demonstrate VHHs according to the invention which bind TNF-alpha and moreover, block its binding to the TNF-alpha receptor. Oral delivery of these anti-TNF-alpha VHH results in the delivery of such molecules in an active form in the colon at sites that are affected by the disease. These sites are highly inflamed and contain TNF-alpha-producing cells. These anti-TNF-

alpha VHH can neutralise the TNF-alpha locally, avoiding distribution throughout the whole body and thus limiting negative side-effects. Genetically modified microorganisms such as *Micrococcus lactis* are able to secrete antibody fragments. Such modified microorganisms can be used as vehicles for local production and delivery of antibody fragments in the intestine. By using a strain which produces a TNF neutralizing antibody fragment, inflammatory bowel disease could be treated. Another aspect of the invention is one or more VHHs specific for TNF-alpha for use in the treatment, prevention and/or alleviation of diseases relating to inflammatory processes, wherein said VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating diseases relating to inflammatory processes, comprising administering to a subject a VHH specific for an antigen related to the disease orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

Further non-limiting examples of therapeutic targets against which the VHHs of the invention may be used are certain colon cancer specific antigens, such as, for example, CEA or EGF receptors. In one aspect of the invention, therapeutic VHHs against colon cancer antigens are linked to one more tumor destroying reagents such as for example, a chemical compound or a radioactive compound.

As stated above a colon cancer specific antigen according to the invention is epidermal growth factor receptor (EGFr) which is an essential mediator of cell division in mammalian cells and is a recognised cellular oncogene. After the binding of EGF to its receptor (EGFr), a signaling cascade is initiated resulting in cell development. The EGFr is also involved in human tumorigenesis as it is overexpressed on cells associated with epithelial malignancies located in sites such as the head, neck, lung, colon. Another aspect of the invention is one or more VHHs specific for TNF for use in the treatment, prevention and/or alleviation of diseases relating to EGFr-mediated cancer, wherein said VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating diseases relating to EGFr-mediated cancer, comprising administering to a subject a VHH specific for an antigen related to the disease orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

As stated above another colon cancer specific antigen according to the invention is carcinoembryonic antigen (CEA), a recognized tumor marker. Another aspect of the invention is one or more VHHs specific for CEA for use in the treatment, prevention and/or

alleviation of diseases relating to CEA-mediated cancer, wherein said VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating diseases relating to CEA-mediated cancer, comprising administering to a subject a VHH specific for an antigen related to the disease orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. A few VHHs specific for this glycoprotein have been isolated by selection on solid-phase coated with CEA out of a dedicated library obtained after immunization of a dromedary. By using FACS analysis it appeared that only two fragments recognized the cell-bound antigen. One of the VHHs, that recognised the native structure, has been used to construct a fusion protein with β-lactamase. The functionality of the purified fusion protein was tested *in vitro* in a prodrug converting cytotoxicity assay. In addition the immunoconjugate was tested *in vivo* in a tumor-targeting biodistribution study.

A non-limiting example of a therapeutic target against which the VHHs of the invention may be used is *Helicobacter pylori*, which is a bacterium that lives in the mucus which coats the lining of the human stomach and duodenum. The normal human stomach has a very thin layer of mucus that coats the whole of its inside surface. This mucus has a protective role, acting as a barrier between the acid in the stomach and the sensitive stomach wall. *H. pylori* acts as an irritant to the lining of the stomach, and this causes inflammation of the stomach (gastritis). In one embodiment of the invention the VHH is specific for *H. pylori* and inhibits the enzymatic function of urease. Since VHH molecules have the specific characteristic to occupy enzymatic sites, selected VHHs would inhibit the enzymatic activity and neutralize the virulence of a *H. pylori* infection. In another aspect of the invention VHH is specific for *H. pylori* and inhibits the adhesion of the bacteria to the stomach wall so preventing irritation of the stomach wall and gastritis. One aspect of the invention is one or more VHHs specific for *Helicobacter pylori* for use in the treatment, prevention and/or alleviation of diseases relating to irritation of the stomach wall and gastritis, wherein said VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation, but preferably orally. Another aspect of the invention is a method of treating, preventing and/or alleviating diseases relating to irritation of the stomach wall and gastritis, comprising administering to a subject a VHH specific for an antigen related to the disease orally, sublingually, topically, nasally, vaginally, rectally or by inhalation, but preferably orally.

Another non-limiting example of a therapeutic target against which the VHH of the invention may be used is HIV. According to the invention, one or more therapeutic VHHs

are delivered to the vaginal and/or rectal tract so as to reduce transmission of HIV. In one aspect of the invention, the VHH is directed against a neutralizing viral epitope that inhibits infection of cells (gp120/gp41). In another aspect of the invention, the VHH is specific for a viral protein involved in viral replication. One aspect of the invention is one or more VHHs specific an HIV epitope such as described above for use in the treatment, prevention and/or alleviation of diseases relating to AIDS, wherein said VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating diseases relating to AIDS, comprising administering to a subject said VHH orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

Another non-limiting example of a therapeutic target against which the VHH of the invention may be used is Hepatitis E, which is a viral disease transmitted via the fecal/oral route. Symptoms increase with age and include abdominal pain, anorexia, dark urine, fever, hepatomegaly, jaundice, malaise, nausea, and vomiting. The overall fatality rate is 1-3%, but 15-25% in pregnant women. Once encountered, most patients develop a neutralizing IgG response which gives life-long protection Neutralizing VHH molecules have the advantage over conventional IgG molecules because they may be administered orally. Since most infections with hepatitis E occur in North-Africa, Central-Africa, Asia and Central-America, oral administration is a significant advantage, since medical logistics are less developed in those countries. One aspect of the invention is one or more VHHs specific for HEV capsid protein (56kDa) for use in the treatment, prevention and/or alleviation of diseases relating hepatitis E, wherein said VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating diseases relating to hepatitis E, comprising administering to a subject said VHH orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

Other non-limiting examples of therapeutic targets against which the VHH of the invention may be used are respiratory ones such as the TB bacterium and influenza virus. TB or tuberculosis, is a disease caused by bacteria called *Mycobacterium tuberculosis.* The bacteria can attack any part of the body, but they usually attack the lungs. Influenza is a viral disease that causes 'flu'. Influenza viruses are also present in the lung. One aspect of the invention is one or more VHHs specific for a *Mycobacterium tuberculosis* epitope for use in the treatment, prevention and/or alleviation of diseases relating TB, wherein said VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or

alleviating diseases relating to TB, comprising administering to a subject said VHH orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is one or more VHHs specific for an influenza virus epitope for use in the treatment, prevention and/or alleviation of diseases relating flu, wherein said VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating diseases relating to flu, comprising administering to a subject said VHH orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

Another non-limiting example of a therapeutic target against which the VHH of the invention may be used is IgE in relation to allergies. During their lifetime, several subjects develop an allergic response to harmless parasites (e.g. Dermatophagoides pteronyssinus, house dust mite) or substances (clumps, plastics, metals). This results in the induction of IgE molecules that initiate a cascade of immunological responses. One aspect of the present invention is one or more IgE-specific VHH molecules that prevent the interaction of IgE with their receptor(s) on mast cells and basophils. As such they prevent the initiation of the immunological cascade, an allergic reaction. Since IgE molecules are present in the bloodstream, it is within the scope of the invention to fuse the VHH one or more active transport carriers in order to reach their target. Another aspect of the invention is one or more VHHs specific an IgE epitope for use in the treatment, prevention and/or alleviation of diseases relating to allergies, wherein said VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating diseases relating to allergies, comprising administering to a subject said VHH orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

Another non-limiting example of a therapeutic target against which the VHH of the invention may be used is human macrophage elastase (MMP-12), which is a member of the family of matrix metalloproteases (MMPs). These enzymes play an important role in normal and inflammatory processes contributing to tissue remodeling and destruction. MMP-12 is secreted into the extracellular space by lung alveolar macrophages and dysregulation of MMP-12 is a possible reason for degradation of the alveolar membrane leading to lung emphysema. Target substrates of MMP-12 include extracellular matrix proteins such as elastin, fibronectin and laminin, but also α1-antitrypsin and tissue factor protease inhibitor. One aspect of the invention is one or more VHHs specific MMP-12 for use in the treatment, prevention and/or alleviation of diseases relating to inflammatory processes, wherein said VHH is administered orally, sublingually, topically, nasally,

vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating diseases relating to inflammatory processes, comprising administering to a subject said VHH orally, sublingually,. topically, nasally, vaginally, rectally or by inhalation.

Another aspect of the present invention is a method to determine which VHH molecules are actively transported into the bloodstream upon oral administration. In a non-limiting example, the method comprises administering orally a naïve or immune VHH phage library to a small animal such as a mouse. At different time points after administration, blood is retrieved to rescue phages that have been actively transferred to the bloodstream. Additionally, after oral administration, organs can be isolated and bound phages can be stripped off. The method of the invention thus identifies VHH which are not only actively transported to the blood, but are also able to target specific organs. One aspect of the invention includes said VHH molecules. According to the invention, said VHH may be used as a carrier by fusing it to a therapeutic VHH directed to a corresponding target in the bloodstream upon oral administration.

Another aspect of the present invention is a method to determine which VHH molecules are actively transported into the bloodstream upon nasal administration Similarly, a naïve or immune VHH phage library can be administered nasally, and after different time points after administration, blood or organs can be isolated to rescue phages that have been actively transported to the bloodstream. A non-limiting example of a receptor for active transport from the lung lumen to the bloodstream is the Fc receptor N (FcRn). One aspect of the invention includes the VHH molecules identified by the method. Such VHH can then be used as a carrier VHH for the delivery of a therapeutic VHH to the corresponding target in the bloodstream upon nasal administration.

Another non-limiting example of a therapeutic target against which the VHH of the invention may be used is IFN-gamma which is secreted by some T cells. In addition to its anti-viral activity, IFN gamma stimulates natural killer (NK) cells and T helper 1 (Th1) cells, and activates macrophages and stimulates the expression of MHC molecules on the surface of cells. Hence, IFN gamma generally serves to enhance many aspects of immune function, and is a candidate for treatment of disease states where the immune system is over-active (e.g. Crohn's disease), e.g., autoimmune diseases and organ plant rejection. One aspect of the invention is one or more VHHs specific IFN for use in the treatment, prevention and/or alleviation of diseases relating to the immune response, wherein said VHH is administered orally, sublingually, topically, nasally, vaginally, rectally

or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating diseases relating to the immune response, comprising administering to a subject said VHH orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

Another non-limiting example of a therapeutic target against which the VHH of the invention may be used is CD28. It is now generally accepted that to activate a T cell, both an antigen-specific and antigen-nonspecific signal must be provided by an activating or antigen presenting cell (APC). The antigen-specific signal is mediated by 1) antigen bound to a class II (or I) MHC molecule on the APC, and 2) the T cell receptor (TCR)/CD4 (or CD8) complex on T cells. While the effect of this ligation is to initiate IL-2 production and IL-2 receptor (IL-2 R) expression by the T cell, these results are self-limiting unless they are amplified by a co-stimulus. This co-stimulus is provided by B7-1 binding to CD28, which stabilises IL-2 mRNA and increases IL-2 secretion, resulting in T cell proliferation and clonal expansion. For the T cell, this two-signal combination has led to three possible outcomes relative to the engagement of the TCR complex and CD28 antigen. If both the TCR and CD28 are ligated, the T cell proliferates and is primed to become one of a limited number of functional subtypes. If only the TCR is ligated, the T cell enters either a state of anergy or undergoes apoptosis. If only the CD28 antigen is engaged, there is no effect. Thus, the reinforcing activity of CD28 seems essential to normal T cell activation. VHH molecules directed against the CD28 molecule would prevent the T cell activation and serve as a therapeutic in the treatment of inflammatory and autoimmune diseases. One aspect of the invention is one or more VHHs specific CD28 for use in the treatment, prevention and/or alleviation of diseases relating to the inflammation and autoimmunity, wherein said VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating diseases relating to inflammation and autoimmunity, comprising administering to a subject said VHH orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

BRIEF DESCRIPTION OF FIGURES AND TABLES

Figure 1: The results of experiments to determine the inhibitory effect of VHH upon the binding of vWF to collagen as in Example 7.

Figure 2: The results of experiments to determine the inhibitory effect of VHH upon the binding of vWF to the platelet receptor gpIb as in Example 8.

Figure 3: Western blot for expression of the A1 and A3 domain of vWF on the surface of *E.coli* as a fusion with OprI.

Figure 4: Agarose gel of DNAse digest of vWF fragment (aa 1371 to 1985) with decreasing concentrations of DNAse.

Figure 5: Results of sequencing A1 or A3 specific binders according to Example 6.

Figure 6. The sequence of the A3 domain of vWF.

Figure 7. Binding in ELISA to vWF for C37 stored at -20°C as compared to C37 incubated at 37°C for 196 hours.

Figure 8. Residual activity for C37 stored at -20°C as compared to C37 incubated at 37°C for up to 194 hours. C37 stability is compared to stability of a scFv specific for B3 antigen and a stabilised form, dsFv (stabilised by 2 disulphide bonds).

Figure 9. Half life of VHH versus that of conventional antibodies (IgG2 and IgG3) upon injection in mouse.

Figure 10. Phage ELISA for vWF, Idomain of gpIa/IIa, gpIb, collagen and casein (negative control) for VHH libraries of llama 002 or llama 004 PBL (first and second bleeding) versus lymph node punctions.

Figure 11. Binding of purified C37, A50 or A38 to vWF measured by ELISA.

Figure 12. Schematic illustrating the regions of IgE.

Figure 13. The sequences obtained after sequencing clones which were positive for human and chimaeric IgE binding.

Figure 14. The relative alignment of the sequences of Figure 13.

Figure 15. Alignment of anti-TNF VHH's as described in Example 15

Figure 16. Dilution series of anti-TNF VHHs as tested in ELISA according to Example 15.

Figure 17. Antagonistic effect of VHH as determined in cytotoxic assay according to Example 15.

Figure 18: Schematic illustration of the region obtained by PCR from the experiment of Example 16.

Figure 19: Agarose gel electrophoresis pattern of the PCR from the experiment of Example 16.

Table 1: Amino acid sequence listing of the peptides of aspects of present invention directed against human von Willebrand factor (vWF) and of human von Willebrand factor itself. The sequence of human vWF indicates A1 and A3 domains respectively in bold lettering.

TABLE 1-1

NAME	SEQ ID NO	SEQUENCE
C37	1	QVQLQESGGGLVQPGGSLRLSCAASGFNFNWYPMSWVRQAPGKGLEWVSTISTYGEPRY ADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYYCARGAGTSSYLPQRGNWDQGTQVT ISS
T76	2	QVQLQESGGGLVQPGESLRLSCAASGSIFSINTMGWYGQAPGKQRELVASITFGGVTNY ADSVKGRFTISRDNTNDTVYLQMNSLKPEDTAVYICNAVTWGGLTNYWGQGTQVTVSS
Z29	3	QVQLQESGGGSVQAGDSLTLSCAASGRTFSMHAMGWFRQAPGKEREFVAAISPSAFTTE YADSLKGRFTVSRDNAKKLVWLQMNGLKPEDTAAYYCAARRGAFTATTAPLYDYWGQGT QVTVSS
A50	4	QVQLQESGGGLVQAGGSLRLSCAASGRTFSSYRMGWFRQAPGKEREFVAAISRRGDNVY YADSVKGRFAISRDNAESTLYLQMNSLKPEDTAVYYCAAHVTVSAITLSTSTYDYWGQG TQVTVSS
A38	5	QVQLQDSGGGSVQAGGSLRLSCAASGRTVSSYNMGWFRRVPGKERDFVAAISWSGVATY YFDSVKGRFTISRDNAKNTVYLEMNSLKPEDTAVYYCAAASRYRHRLNSGSEYDYWGQG TQVTVSS
I53	6	QVQLQDSGGGLVQAGGSLRLSCAASGRTKDMAWFRQPPGKEREFVAVIYSSDGSTLVAA SVKGRFTISRDNAKNTVYLQMTSLKPADTAVYYCATSRGYSGTYYSTSRYDYWTGGTQV TVSS
M53	7	QVQLQDSGGGLVQAGESLRLSCGTSGRTFGRRAMAWFRQAPGKERQFVAWIARYDGSTL YADSVKGRFTISRDDNKNTMYLHMNNLTPEDTAVYYCAAGPRGLYYESRYEYWGQGTLV TVSS
2L-34	8	QVQLQDSGGGLVQAGGSLRLSCAASVRIFTSYAMGWFRQAPGKEREFVA AINRSGKSTYYSDSVEGRFTISRDNAKNTVSLQMDSLKLEDTAVYYCAA DYSGSYTSLWSRPERLDWGQGTQVTVFS
4L-16	9	QVQLVESGGGLVQAGGSLRLSCAASGRTFSSYAMGWFRQAPGKEREFVA AISWSGGSTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCVA DTGGISWIRTQGYNYWGQGTQVTVSS
Human vWF	10	MIPARFAGVLLALALILPGTLCAEGTRGRSSTARCSLFGSDFVNTFDGSMYSFAGYCSY LLAGGCQKRSFSIIGDFQNGKRVSLSVYLGEFFDIHLFVNGTVTQGDQRVSMPYASKGL YLETEAGYYKLSGEAYGFVARIDGSGNFQVLLSDRYFNKTCGLCGNFNIFAEDDFMTQE GTLTSDPYDFANSWALSSGEQWCERASPPSSSCNISSGEMQKGLWEQCQLLKSTSVFAR CHPLVDPEPFVALCEKTLCECAGGLECACPALLEYARTCAQEGMVLYGWTDHSACSPVC PAGMEYRQCVSPCARTCQSLHINEMCQERCVDGCSCPEGQLLDEGLCVESTECPCVHSG KRYPPGTSLSRDCNTCICRNSQWICSNEECPGECLVTGQSHFKSFDNRYFTFSGICQYL LARDCQDHSFSIVIETVQCADDRDAVCTRSVTVRLPGLHNSLVKLKHGAGVAMDGQDIQ LPLLKGDLRIQHTVTASVRLSYGEDLQMDWDGRGRLLVKLSPVYAGKTCGLCGNYNGNQ GDDFLTPSGLAEPRVEDFGNAWKLHGDCQDLQKQHSDPCALNPRMTRFSEEACAVLTSP TFEACHRAVSPLPYLRNCRYDVCSCSDGRECLCGALASYAAACAGRGVRVAWREPGRCE LNCPKGQVYLQCGTPCNLTCRSLSYPDEECNEACLEGCFCPPGLYMDERGDCVPKAQCP CYYDGEIFQPEDIFSDHHTMCYCEDGFMHCTMSGVPGSLLPDAVLSSPLSHRSKRSLSC RPP

TABLE 1-2

Human vWF Cont...		MVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCVALERCPCF HQGKEYAPGETVKIGCNTCVCRDRKWNCTDHVCDATCSTIGMAHYLTFDGLKYLFPGECQ YVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKKRVTILVEGGEIELFDGEVNVKRPMKDE THFEVVESGRYIILLLGKALSVVWDRHLSISVVLKQTYQEKVCGLCGNFDGIQNNDLTSS NLQVEEDPVDFGNSWKVSSQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRILTSDVFQD CNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAAYAHVCAQHGKVVTWRTATLCPQSC EERNLRENGYECEWRYNSCAPACQVTCQHPEPLACPVQCVEGCHAHCPPGKILDELLQTC VDPEDCPVCEVAGRRFASGKKVTLNPSDPEHCQICHCDVVNLTCEACQEPGGLVVPPTDA PVSPTTLYVEDISEPPLHDFYCSRLLDLVFLLDGSSRLSEAEFEVLKAFVVDMMERLRIS QKWVRVAVVEYHDGSHAYIGLKDRKRPSELRRIASQVKYAGSQVASTSEVLKYTLFQIFS KIDRPEASRIALLLMASQEPQRMSRNFVRYVQGLKKKKVIVIPVGIGPHANLKQIRLIEK QAPENKAFVLSSVDELEQQRDEIVSYLCDLAPEAPPPTLPPHMAQVTVGPGLRNSMVLDV AFVLEGSDKIGEADFNRSKEFMEEVIQRMDVGQDSIHVTVLQYSYMVTVEYPFSEAQSKG DILQRVREIRYQGGNRTNTGLALRYLSDHSFLVSQGDREQAPNLVYMVTGNPASDEIKRL PGDIQVVPIGVGPNANVQELERIGWPNAPILIQDFETLPREAPDLVLQRCCSGEGLQIPT LSPAPDCSQPLDVILLLDGSSSFPASYFDEMKSFAKAFISKANIGPRLTQVSVLQYGSIT TIDVPWNVVPEKAHLLSLVDVMQREGGPSQIGDALGFAVRYLTSEMHGARPGASKAVVIL VTDVSVDSVDAAADAARSNRVTVFPIGIGDRYDAAQLRILAGPAGDSNVVKLQRIEDLPT MVTLGNSFLHKLCSGFVRICMDEDGNEKRPGDVWTLPDQCHTVTCQPDGQTLLKSHRVNC DRGLRPSCPNSQSPVKVEETCGCRWTCPCVCTGSSTRHIVTFDGQNFKLTGSCSYVLFQN KEQDLEVILHNGACSPGARQGCMKSIEVKHSALSVELHSDMEVTVNGRLVSVPYVGGNME VNVYGAIMHEVRFNHLGHIFTFTPQNNEFQLQLSPKTFASKTYGLCGICDENGANDFMLR DGTVTTDWKTLVQEWTVQRPGQTCQPILEEQCLVPDSSHCQVLLLPLFAECHKVLAPATF YAICQQDSCHQEQVCEVIASYAHLCRTNGVCVDWRTPDFCAMSCPPSLVYNHCEHGCPRH CDGNVSSCGDHPSEGCFCPPDKVMLEGSCVPEEACTQCIGEDGVQHQFLEAWVPDHQPCQ ICTCLSGRKVNCTTQPCPTAKAPTCGLCEVARLRQNADQCCPEYECVCDPVSCDLPPVPH CERGLQPTLTNPGECRPNFTCACRKEECKRVSPPSCPPHRLPTLRKTQCCDEYECACNCV NSTVSCPLGYLASTATNDCGCTTTTCLPDKVCVHRSTIYPVGQFWEEGCDVCTCTDMEDA VMGLRVAQCSQKPCEDSCRSGFTYVLHEGECCGRCLPSACEVVTGSPRGDSQSSWKSVGS QWASPENPCLINECVRVKEEVFIQQRNVSCPQLEVPVCPSGFQLSCKTSACCPSCRCERM EACMLNGTVIGPGKTVMIDVCTTCRCMVQVGVISGFKLECRKTTCNPCPLGYKEENNTGE CCGRCLPTACTIQLRGGQIMTLKRDETLQDGCDTHFCKVNERGEYFWEKRVTGCPPFDEH KCLAEGGKIMKIPGTCCDTCEEPECNDITARLQYVKVGSCKSEVEVDIHYCQGKCASKAM YSIDINDVQDQCSCCSPTRTEPMQVALHCTNGSVVYHEVLNAMECKCSPRKCSK

Table 2: Amino acid sequence listing of the peptides of aspects of present invention directed against TNF-alpha.

TABLE 2

NAME	SEQ ID NO	SEQUENCE
VHH#1	11	QVQLQESGGGLVQPGGSLRLSCATSGFDFSVSWMYWVRQAPGKGLEWVSEINTNGLITKY VDSVKGRFTISRDNAKNTLYLQMDSLIPEDTALYYCARSPSGSFRGQGTQVTVSS
VHH#9	12	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFVAIITSGDNLNYA DAVKGRFTISTDNVKKTVYLQMNVLKPEDTAVYYCNAILQTSRWSIPSNYWGQGTQVTVS S
VHH#13	13	QVQLQESGGGLVQPGGSLRLSCATSGFTFSDYWMYWVRQAPGKGLEWVSTVNTNGLITRY ADSVKGRFTISRDNAKYTLYLQMNSLKSEDTAVYYCTKVVPPYSDDSRTNADWGQGTQVT VSS
VHH#2	14	QVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGKEREFVARIYWSS GNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPEDTAVYYCAARDGIPTSRSVESYNYWG QGTQVTVSS
VHH#3	15	QVQLQDSGGGLVQAGGSLRLSCAVSGRTFSAHSVYTMGWFRQAPGKEREFVARIYWSSAN TYYADSVKGRFTISRDNAKNTVDLLMNSLKPEDTAVYYCAARDGIPTSRTVGSYNYWGQG TQVTVSS
VHH#4	16	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFVAIITSSDTNDTT NYADAVKGRFTISTDNVKKTVYLQMNVLKPEDTAVYYCNAVLQTSRWSIPSNYWGQGTQV TVSS
VHH#5	17	QVQLQDSGGGLVQAGGSLRLSCTTSGRTISVYAMGWFRQAPGKEREFVASISGSGAITPY ADSVKGRFTISRDNAKNTVYLQMNSLNPEDTAVYYCAASRYARYRDVHAYDYWGQGTQVT VSS
VHH#6	18	QVQLQDSGGGLVQAGGSLRLSCAASTRTFSRYVVGWFRQAPGKEREFVATISWNGEHTYY ADSVKGRYTISRDNAKNTVYLQMGSLKPEDTAVYYCAARSFWGYNVEQRDFGSWGQGTPV TVSS
VHH#7	19	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFVAIITNDTTNYAD AVKGRFTISTDNVKKTVYLQMNVLKPEDTAVYYCNTVLQTSRWNIPTNYWGQGTQVTVSS
VHH#8	20	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFVAIISGDTTNYAD AVKGRFTISTDNVKKTVYLQMNVLESEDTAVYYCNAVLQTSRWSIPSNYWGQGTQVTVSS
VHH#10	21	QVQLQDSGGGLVQPGGSLRLACVASGSIFSIDVMGWYRQAPGQQRELVATITNSWTTNYA DSVKGRFTISRDNAKNVVYLQMNSLKLEDTAVYYCNARRWYQPEAWGQGTQVTVSS
VHH#11	22	QVQLQDSGGGLVQPGGSLRLSCAASGFTFSTHWMYWVRQAPGKGLEWVSTINTNGLITDY IHSVKGRFTISRDNAKNTLYLQMNSLKSEDTAVYYCALNQAGLSRGQGTQVTVSS
VHH#12	23	QVQLQESGGGLVQAGGSLRLSCAASRRTFSGYAMGWFRQAPGKEREFVAVVSGTGTIAYY ADSVKGRFTISRDNAENTVYLQMNSLKPEDTGLYYCAVGPSSSRWYYRGASLVDYWGKGT LVTVSS

EXAMPLES

The invention is illustrated by the following non-limiting examples.

Materials

Human placental collagen type VIII (also known as collagen type I) was purchased from Sigma (St. Louis, MO).

Collagen was solubilized in 50 mM acetic acid at 5mg/ml, diluted with PBS to 1mg/ml and dialysed against PBS (phosphate buffered saline) to obtain fibrillar collagen.

vWF was purchased from the Red Cross (Belgium). Anti-mouse IgG alkaline phosphatase conjugate was obtained from Sigma. Mouse anti-histidin tag was purchased from Serotec. HRP- anti-Von Willebrand Factor conjugate was obtained from DAKO. HRP anti-M13 monoclonal conjugate was purchased from Amersham Biosciences.

Example 1: Immunization of llamas

2 llamas were immunized with a cocktail of vWF, platelet receptor gpIb, platelet receptor gpIa/IIa (α_2I-domain) and collagen. The immunization schemes are summarized in Tables 3 and 4.

Table 3: Immunisation schemes used for llama 002 according to Example 1, wherein rgpIb is the platelet receptor gpIb.

Llama002 Day of immunization	vWF	rgpIb	gpIa/Ia α_2I-domain	Collagen
0	100 µg	40 µg	40 µg	100 µg
7	100 µg	40 µg	40 µg	100 µg
14	50 µg	20 µg	20 µg	50 µg
21	50 µg	20 µg	20 µg	50 µg
28	50 µg	20 µg	20 µg	50 µg
35	50 µg	20 µg	20 µg	50 µg

Table 4: Immunisation schemes used for llama 004 according to Example 1, wherein rgpIb is the platelet receptor gpIb.

Llama004 Day of immunization	vWF	rgpIb	gpIa/Ia α_2I-domain	Collagen
0	100 µg	40 µg	40 µg	100 µg
21	100 µg	40 µg	40 µg	100 µg
42	50 µg	20 µg	20 µg	50 µg
70	50 µg	20 µg	20 µg	50 µg

Example 2: Repertoire cloning

Different sources for RNA extraction were used:

- 150 ml immune blood, between 4 and 10 days after the last antigen injection
- lymph node biopsy 4 days after the last antigen injection.

Peripheral blood lymphocytes (PBLs) were isolated by centrifugation on a density gradient (Ficoll-Paque Plus Amersham Biosciences). PBLs and lymph node were used to extract total RNA (Chomczynski and Sacchi 1987) followed by synthesis of cDNA using a hexanucleotide random primer. The repertoire was amplified using two hinge-specific primers:

AACAGTTAAGCTTCCGCTTGCGGCCGCGGAGCTGGGGTCTTCGCTGTGGTGCG and AACAGTTAAGCTTCCGCTTGCGGCCGCTGGTTGTGGTTTTGGTGTCTTGGGTT and a framework 1 specific primer: GAGGTBCARCTGCAGGASTCYGG. Fragments were digested with PstI and NotI and cloned into a phagemid vector. The repertoire was transformed in TG1 electrocompetent cells and plated on LB agar plates containing 100 µg/ml ampicillin and 2% glucose. Colonies were screened for the presence of insert by PCR with vector specific primers. Results are summarized in Table 5.

Table 5: Results of cloning of VHH repertoire in a phagemid vector for llama002 and llama004 with different sources of RNA and different time points after the last immunisation.

	#days after last injection	Source RNA	Size of the library	% insert
Llama002	4	Lymph	1.3×10^7	89
	7	PBL	1.9×10^7	95
	10	PBL	1.1×10^9	70
Llama004	4	PBL	1.7×10^8	96
	4	Lymph	4.9×10^7	>95

Example 3: Rescue of the library, phage preparation

Libraries were grown at 37°C in 60 ml 2xTY medium containing 2% glucose, and 100 µg/ml ampicillin, until the OD600nm reached 0.5. M13KO7 phages (1012) were added and the mixture was incubated at 37°C for 2 x 30 minutes, first without shaking, then with shaking at 100 rpm. Cells were centrifuged for 10 minutes at 4500 rpm at room temperature. The bacterial pellet was resuspended in 300 ml of 2xTY medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin, and incubated overnight at 30°C with vigorously shaking at 250 rpm. The overnight cultures were centrifuged for 15 minutes at 10.000 rpm at 4°C. Phages were PEG precipitated (20% poly-ethylene-glycol and 1.5 M

NaCl) and centrifuged for 30 minutes at 10.000 rpm. The pellet was resuspended in 20 ml PBS. Phages were again PEG precipitated and centrifuged for 30 minutes at 20,000 rpm and 4°C. The pellet was dissolved in 5 ml PBS. Phages were titrated by infection of TG1 cells at OD600nm= 0.5 and plating on LB agar plates containing 100 µg/ml ampicillin and 2% glucose. The number of transformants indicates the number of phages (= pfu). The phages were stored at –80°C with 15% glycerol.

Example 4: Phage ELISA

A microtiter plate (Maxisorp) was coated overnight at 4°C with PBS-1% casein, 2 µg/ml vWF, 10 µg/ml gpIb or 25 µg/ml gpIa/Ia α_2I-domain in carbonate buffer containing 5 mM Mg Cl_2, or coated overnight at 4°C with 1% casein and 25µg/ml collagen in PBS. The plate was washed 3 times with PBS-Tween (0.05% Tween20) and blocked for 2 hours at room temperature with 200 µl PBS-1% casein. The plate was washed five times with PBS-Tween. Phages were prepared as described above and applied to the wells in duplo dilutions. Plates were washed five times with PBS-Tween. Binding phages were detected with a mouse anti-M13 conjugated with HRP diluted 1/2000 in PBS. The plates were washed five times with PBS-Tween. Staining was performed with ABTS/H2O2 and signals were measured after 30 minutes at 405 nm. The results are presented in Figure 10. It is clear from the results that specific VHH are present in all libraries for all the different antigens.

Example 5: Selection

Immunotubes were coated with 2 µg/ml vWF or with PBS containing 1% casein. After overnight incubation at 4°C, the tubes were blocked with PBS containing 1% casein, for 3 hours at RT. 200 µl phages of the three libraries of llama002 were pooled and added to the immunotubes with a final volume of 2 ml in PBS. After 2 hours incubation at RT, the immunotubes were washed 10x with PBS-Tween and 10x with PBS. Bound phages were eluted with 2 ml 0.2 M glycin buffer pH= 2.4 or with 2 ml of a 100 µg/ml collagen solution. Elutions were performed for 20 minutes or 1 hour respectively at room temperature. Alternatively, 200 µl phages of llama004 were added to the immunotubes and bound phages were eluted with 100 µg/ml collagen solution. Eluted phages were allowed to infect exponentially growing TG1 cells, and were then plated on LB agar plates containing 100 µg/ml ampicillin and 2% glucose. The results from the panning are presented in Table 6.

Table 6: Plaque forming units (pfu) after one round of panning on vWF for llama002 and llama004, with different elution conditions. Pfu vWF (antigen) divided by pfu casein (aspecific binding) = enrichment.

llama	Source RNA	Elution conditions	Pfu vWF	Pfu casein	Enrichment
002	Pool of the 3 libraries	0.2 M glycin, pH 2.4	1.5×10^7	1×10^4	1.500
002	Pool of the 3 libraries	100 µg/ml collagen	4.5×10^6	3.2×10^4	140
004	PBL day 4	100 µg/ml collagen	1.8×10^5	250	720
004	Lymph day 4	100 µg/ml collagen	3.6×10^5	250	1.440

Example 6: Screening

Cloning A1 and A3 domain of vWF in pBAD-OprI-ss

The pBAD-OprI-strep-spec vector was used to display the VWF A1 and A3 domains as a fusion with OprI on the surface of UT5600 *E.coli* cells (F- ara-14 leuB6 azi-6 lacY1 proC14 tsx-67 entA403 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi1 DompT fepC266) (Cote-Sierra et al, 1998, *Gene*, 221: 25-34). The gene coding for the A1 domain of vWF (219aa) was amplified by PCR using the A1for and A1back PCR primers. The gene coding for the A3 domain of vWF (201aa) was amplified by PCR using the A3for and A3back PCR primers.

A1for: CCG GTG AGC CCC ACC ACT CTA AGC TTG GAG GAC ATC TCG GAA CCG
A1back: CCC CAG GGT CGA AAC CCT CTA GAG CCC CGG GCC CAC AGT GAC
A3for: CTG GTG CTG CAG AGG TGA AGC TTC GGA GAG GGG CTG CAG ATC
A3back: ATC CAT GCA AAT CCT CTA GAA TCC AGA GCA CAG TTT GTG GAG

Fragment and vector were digested with HindIII and XbaI, ligated and transformed in UT5600 (= pBAD-vWFA1/pBAD-vWFA3). Transformed cells were plated on LB agar plates containing 20 µg/ml streptomycin, 50 µg/ml spectinomycin.

The pBAD-vWFA1/pBAD-vWFA3 plasmids were transformed in UT5600 F- cells and plated on LB agar plates with 20 µg/ml streptomycin, 50 µg/ml spectinomycin. A single colony was used to inoculate LB medium with 20 µg/ml streptomycin, 50 µg/ml spectinomycin. Cells were grown overnight at 37°C at 200 rpm. The next day, cells were induced with 0.2% arabinose and incubated for 1 more hour at 37°C at 150 rpm. Total cell lysates were boiled in reducing sample buffer, loaded on a 12% SDS-PAGE and transferred to nitrocellulose for Western blotting. Transferred proteins were detected using a monoclonal anti-OprI antibody (SH2.2) (Cote-Sierra et al, 1998, *Gene*, 221: 25-34). An

anti-mouse IgG conjugated with alkaline phosphatase was applied (Sigma), and the blots were developed with BCIP/NBT (Figure 3).

ELISA: binding of phage expressing VHH to *E.coli* cells expressing the A1 or A3 domain on their surface.

The pBAD-vWFA1/pBAD-vWFA3 plasmids were transformed in UT5600 F- cells and plated on LB agar plates with 20 µg/ml streptomycin, 50 µg/ml spectinomycin. A single colony was used to inoculate LB medium with 20 µg/ml streptomycin, 50 µg/ml spectinomycin. Cells were grown overnight at 37°C at 200 rpm. The next day, cells were induced with 0.2% arabinose and incubated for 1 more hour at 37°C at 150 rpm. A microtiter plate was coated overnight at 4°C with the monoclonal anti-OprI antibody (SH2.2) diluted 1/1000 in PBS. After induction, total cells were allowed to bind to the plate for 1 hour at room temperature. The plates were washed five times with PBS-Tween. Phage preparations of single colonies of the different VHHs were allowed to bind for two hours at room temperature. The plates were washed five times with PBS-Tween. An anti-M13 HRP conjugate was used for detection of phage binding to *E. coli* cells expressing the A1 or A3 domain on their surface. The plates were washed five times with PBS-Tween. Staining was performed with ABTS/H2O2 and signals were measured after 30 minutes at 405 nm. The number of positive clones for each phage preparation is presented in Table 6. A1 or A3 specific binders were sequenced. Sequenced results revealed the presence of 5 different binders for the A1 domain of vWF isolated from llama 002, 1 specifc binder for the A3 domain of the vWF from llama 002 (C37, 2L-34, 4L-16) and from llama 004 (T76). The results of sequencing A1 or A3 binders are given in Figure 5.

Table 7: The results of screening in ELISA of individual colonies for binding to vWF, A1 domain or A3 domain for each selection method.

llama	Elution Buffer	No. clones +ve for vWF / No. tested	No. clones +ve for A1 / No. tested	No. clones +ve for A3 / No. tested
002	0.2M glycine, pH 2.4	344/380	5/570	2/380
002	100 µg/ml collagen	356/380	0/380	3/380
004	100 µg/ml collagen	-	0/96	1/96
004	100 µg/ml collagen	-	0/96	7/96

ELISA: binding to vWF

A microtiter plate was coated with 2 µg/ml vWF, overnight at 4°C. Plates were blocked for two hours at room temperature with 300 µl 1% casein in PBS. The plates were washed three times with PBS-Tween. Dilution series (150 µg/ml to 0.28 ng/ml, dilution factor three) of all purified samples were incubated for 2 hours at RT. Plates were washed six times with PBS-Tween, after which binding of VHH was detected by incubation with mouse anti-Histidine mAB 1/1000 in PBS for 1 hour at RT followed by anti-mouse-alkaline phosphatase conjugate 1/2000 in PBS, also for 1 hour at RT. Staining was performed with the substrate PNPP (p-nitrophenyl-phosphate, 2 mg/ml in 1M diethanolamine, 1mM Mg_2SO_4, pH9.8) and the signals were measured after 30 minutes at 405 nm. The number of positive clones for each selection is presented in Table 7, and the binding as a function of concentration of purified C37, A50 and A38 is indicated in Figure 11.

Example 7: Functional characterization of A1 or A3 binders: Inhibition of binding of vWF to collagen by VHH

A microtiter plate was coated overnight at 4°C with collagen type VIII at 25 µg/ml in PBS. The plate was washed five times with PBS-tween and blocked for 2 hours at room temperature with PBS containing 1% casein. The plate was washed five times with PBS-tween. 60 µl plasma containing vWF (1/20 dilution in PBS-0.1% casein (plasma is incubated at 37°C for 15 minutes)) was mixed with 60 µl periplasmic extract containing a VHH antibody for testing and incubated for 30 minutes at room temperature. 60 µl of this mixture was applied to a well coated with collagen, and incubated for 90 minutes at room temperature. The plate was washed five times with PBS-tween. An anti-vWF-HRP monoclonal antibody was diluted 3,000-fold in PBS and incubated for 1 hour. The plate was washed five times with PBS-tween and vWF-binding was detected with ABTS/H2O2. Signals were measured after 30 minutes at 405 nm. The results presented in Figure 1, demonstrates that C37, 2L-34, 4L-16 and T76 specifically compete with collagen for binding to vWF.

Example 8: Functional characterization of A1 or A3 binders: Inhibition of binding of vWF to the platelet receptor gpIb

A microtiter plate was coated overnight at 4°C with an antibody specific for platelet receptor gpIb at 5µg/ml in PBS. The plate was washed five times with PBS-Tween, and blocked with 300 µl PBS-1% casein for 2 hours at room temperature. The plate was washed 3 times with PBS-Tween. Platelet receptor gpIb (gpIb) was applied to the wells of

the microtiter plate at a concentration of 1 µg/ml and allowed to bind for 2 hours at room temperature. The plate was washed five times with PBS-Tween. Plasma containing vWF was pre-incubated at a dilution of 1/128 at 37°C for 5 minutes. Risto was added at a final concentration of 760 µg/ml and VHH. This mixture was incubated for 30 minutes at room temperature. 100 µl of this mixture was then applied to a microtiter plate well and incubated for 90 minutes at room temperature. The plate was washed five times with PBS-Tween. A anti-vWF-HRP monoclonal antibody was diluted 3.000-fold in PBS and incubated for 1 hour. The plate was washed five times with PBS-tween and vWF-binding was detected with ABTS/H2O2. Signals were measured after 30 minutes at 405 nm. The results presented in Figure 2, demonstrate that A38 and A50 specifically competes with platelet receptor gpIb for binding to vWF.

Example 9: Epitope mapping

A library of fragments of vWF was prepared in pBAD-OprI-ss. Therefore, the gene coding from aa 1371 to aa 1985 (A1-A2-A3 domain of vWF) was amplified by PCR and digested with decreasing concentrations of DNAse (see Figure 4). Fragments were amplified with DNAse primer (AAGCTT(C)17) and XbaI primer (TCTAGA(C)17). Fragment and vector were digested with HindIII and XbaI, ligated and transformed in UT5600 *E.coli* cells. Transformed cells were plated on LB agar plates containing 20 µg/ml streptomycin, 50 µg/ml spectinomycin. The size of the libraries varies between 4 to20 x 104 cfu. Single colonies were grown in 96-well plates and captured on SH2.2-coated ELISA plates. Phages were prepared for a A3 specific VHH (C37) and binding to the OprI fusion expressed DNAse digested vWF fragments was analyzed as described above for A1 and A3. The results indicate the epitope mapped by C37.

Example 10: Expression and purification of VHH

Plasmid was prepared for specific A1 or A3 binders and transformed into WK6 electrocompetent cells. A single colony was used to start an overnight culture in LB containing 2% glucose and 100 µg/ml ampicillin. This overnight culture was diluted 100-fold in 300 µl TB medium containing 100 mg/ml ampicillin, and incubated at 37°C until OD600nm= 0.5. 1 mM IPTG was added and the culture was incubated overnight at 30°C. Overnight cultures were centrifuged for 20 minutes at 10.000 rpm at 4°C. The pellet was resuspended in 10 ml TES (0.2 M Tris-HCl, 0.5 mM EDTA, 0.5 M sucrose, pH= 8.0) and incubated on ice for 20 minutes. 15 ml TES/4 was added and the mixture was incubated for 30 minutes at 4°C. Periplasmic fraction was isolated by centrifugation for 20 minutes at 4°C at 10.000 rpm. The supernatant containing the VHH was loaded on Ni-NTA and

purified to homogeneity. The yield of VHH was calculated according to the extinction coefficient. The results are presented in Table 8.

Table 8: The yields of expression of VHH domains.

VHH code name	Epitope	Extinction coefficient	Yield (mg pure VHH / liter culture)
A50	A1	1.275	5.7
A38	A1	1.671	1.4
C37	A3	1.907	3.3
T76	A3	1.65	1.1
2L-34	A3	1.66	1.5
4L-16	A3	2.29	2.8

Example 11: Stability of VHH at 37°C

VHH C37 was incubated at 37°C and binding activity for vWF was measured at different time points by ELISA as described above. Results were compared to VHH stored at -20°C and are presented in Figure 7.

VHH C37 was incubated at 37°C for up to 3 months and its activity was measured at certain time points. The results are presented in Figure 8. Shown for comparison are the activities of a scFv against B3 antigen (Reiter et al, Protein Engineering, 1994, 7: 697-704), and said scFv modified by the introduction of a disulphide bond between framework residues 44 and 105 to enhance its stability (dsFv), after incubation at 37°C. The results indicate that C37 lost no activity, even after 194 hours at 37°C, while dsFv lost 40% of its activity after 60 hours incubation at 37°C.

Example 12: Experiments to test the polypeptides of the present invention in animal models

The effects of polypeptides represented by SEQ ID NOs: 1 to 9 and other polypeptides, homologous sequences thereof and/or functional portions thereof upon platelet aggregation are being tested using an animal model. Polypeptides are being tested using the methods described in PCT application number WO 02/051351 A2.

Example 13: VHH directed against IgE

Two llama's were immunized with Human IgE, Scripps laboratories, Cat nr. I0224. The following immunization schemes were used according to Table 9.

Table 9.

Day	Llama 2	Llama 4
0	100 µg	100 µg
7	100 µg	
14	50 µg	
21	50 µg	100 µg
28	50 µg	
35	50 µg	
42		50 µg
70		50 µg

Different sources for RNA extraction were used:

- 150 ml immune blood, between 4 and 10 days after the last antigen injection
- lymph node biopsy 4 days after the last antigen injection

Peripheral blood lymphocytes (PBLs) were isolated by centrifugation on a density gradient (Ficoll-Paque Plus Amersham Biosciences). PBLs and lymph node were used to extract total RNA (Chomczynski and Sacchi 1987) followed by synthesis of cDNA using a hexanucleotide random primer. The repertoire was amplified using two hinge-specific primers:

AACAGTTAAGCTTCCGCTTGCGGCCGCGGAGCTGGGGTCTTCGCTGTGGTGCG and AACAGTTAAGCTTCCGCTTGCGGCCGCTGGTTGTGGTTTTGGTGTCTTGGGTT and a framework 1 specific primer:

GAGGTBCARCTGCAGGASTCYGG.

Fragments were digested with PstI and NotI and cloned into a phagemid vector. The repertoire was transformed in TG1 electrocompetent cells and plated on LB agar plates containing 100 µg/ml ampicillin and 2% glucose. Colonies were screened for the presence of insert by PCR with vector specific primers. Results are summarized in Table 10:

Table 10.

	#days after last injection	Source RNA	Size of the library	% insert
Llama002	4	Lymph	1.3×10^7	89
	4	PBL	1.9×10^7	95
	10	PBL	1.1×10^9	70

Llama004	4	PBL	1.7×10^8	96
	4	Lymph	4.9×10^7	>95
	10	PBL	2.2×10^8	>95

Selections were done using chimaeric IgE instead of human IgE, used for immunization, in order to select for VHH molecules directed against the constant region of IgE. The region interacting with the Fcεreceptor is located in the constant part of IgE, more in particular in the region covered by Cε2-Cε3 as shown in Figure 12.

A first selection was performed using the pool of PBL day4, PBL day10 and lymph node day4 libraries for each of the two llama's. Chimaeric IgE was solid phase coated at 5 µg/ml and 0.5 µg/ ml and specific phages were eluted using 0.1 M glycine pH = 2.5. The results obtained are shown in Table 11.

Table 11.

	5 µg/ ml	0.5 µg/ ml	0 µg/ ml (blanco)
Llama 2 (pool PBL day4, PBLday10, lymph node day4)	$1.4\ 10^6$	$2.7\ 10^5$	$1.5\ 10^4$
Enrichment compared to blanco	*400 x*	*18 x*	
Llama 4 (pool PBL day4, PBLday10, lymph node day4)	$3.3\ 10^6$	$4.5\ 10^5$	$7.2\ 10^4$
Enrichment compared to blanco	*140 x*	*6.25 x*	

A second selection was performed using the rescued phages from the first selection using 5 µg/ ml. Chimaeric IgE was solid phase coated at 1 µg/ml and specific phages were eluted using buffy coat cells or lysozyme for 1 hr. Buffy coat cells contain cells expressing the Fcεreceptor, while lysozyme is an irrelevant protein and serves as a control. The results obtained are shown in Table 12:

Table 12.

	1 µg/ ml	1 µg/ ml	0 µg/ ml	0 µg/ ml
	Elution buffy coat cells	Elution Lysozyme	Elution buffy coat cells	Elution Lysozyme

Llama 2 (selection 5 µg/ ml IgE: 400 x enrichment)	$1.2\ 10^8$	$1.2\ 10^8$	$6\ 10^3$	$3\ 10^3$
Enrichment compared to lysozyme elution	No enrichment			2x
Llama 4 (selection 5 µg/ ml IgE: 140 x enrichment)	$1.3\ 10^8$	$2\ 10^7$	$3\ 10^3$	$3\ 10^3$
Enrichment compared to lysozyme elution	6.5 x		No enrichment	

Another second round selection was performed using neutravidine coated tubes and 2 nM biotinylated IgE. Specific phages were eluted using buffy coat cells or lysozyme for 1 hr. Buffy coat cells contain cells expressing the Fcεreceptor, while lysozyme is an irrelevant protein and serves as a control. The results obtained are shown in Table 13:

Table 13.

	2 nM IgE	**2 nM IgE**	**0 nM IgE**	**0 nM IgE**
	Elution buffy coat cells	Elution Lysozyme	Elution buffy coat cells	Elution Lysozyme
Llama 2 (selection 5 µg/ ml IgE: 400 x enrichment)	$1.5\ 10^8$	$1.5\ 10^7$	$3\ 10^5$	$3\ 10^3$
Enrichment compared to lysozyme elution	10 x			
Llama 4 (selection 5 µg/ ml IgE: 140 x enrichment)	$3.3\ 10^7$	$2.2\ 10^7$	$3\ 10^3$	$6\ 10^3$
Enrichment compared to lysozyme elution	1.5 x			

Individual clones obtained from the first round of selection were screened in an ELISA using solid phase coated human IgE or chimaeric IgE. The number of clones that score positive for binding to both human IgE and chimeric IgE versus the number of clones tested in ELISA are summarized in Table 14:

Table 14.

	Selection with 5 µg/ml	Selection with 0.5 µg/ml
Llama 002	39/47	21/47
Llama 004	45/47	46/47

Clones were picked which were positive for human and chimaeric IgE binding, amplified by PCR and digested with HinfI. HinfI profiles were determined on agarose gel and representative clones for different profiles were sequenced. The sequences obtained are shown in Figure 13 and their relative alignment in Figure 14.

Example 14: Example of a new class of antibodies against human tumor necrosis factor alpha

1) Immunization and library constructions

A llama (*Llama glama*) was immunized with human TNF-alpha. For immunization, the cytokine was formulated as an emulsion with an appropriate, animal-friendly adjuvant (Specoll, CEDI Diagnostics B.V.). The antigen cocktail was administered by double-spot injections intramuscularly in the neck. The animal received 6 injections of the emulsion, containing 100 μg of TNF-α at weekly intervals. At different time points during immunization, 10-ml blood samples were collected from the animal and sera were prepared. The induction of an antigen specific humoral immune response was verified using the serum samples in an ELISA experiment with TNF (data not shown). Five days after the last immunization, a blood sample of 150 ml was collected. From this sample, conventional and heavy-chain antibodies (HcAbs) were fractionated (Lauwereys et al. 1998) and used in an ELISA, which revealed that the HcAbs were responsible for the antigen specific humoral immune response (data not shown). Peripheral blood lymphocytes (PBLs), as the genetic source of the llama heavy chain immunoglobulins (HcAbs), were isolated from the 150-ml blood sample using a Ficoll-Paque gradient (Amersham Biosciences) yielding $5x10^8$ PBLs. The maximal diversity of antibodies is expected to be equal to the number of sampled B-lymphocytes, which is about 10 % of the number of PBLs ($5x10^7$). The fraction of heavy-chain antibodies in llama is up to 20 % of the number of B-lymphocytes. Therefore, the maximal diversity of HcAbs in the 150 ml blood sample is calculated as 10^7 different molecules. Total RNA (around 400 µg) was isolated from these cells using an acid guanidinium thiocyanate extraction (Chomczynski and Sacchi, 1987).

cDNA was prepared on 100 µg total RNA with M-MLV Reverse Transcriptase (Gibco BRL) and oligo-dT-primer or hexanucleotide random primers (Amersham Biosciences) as described before (de Haard *et al.*, 1999). The cDNA was purified with a phenol/chloroform extraction combined with an ethanol precipitation and subsequently used as template to specifically amplify the VHH repertoire.

The VHH repertoire was amplified using oligo-dT primed cDNA as template with a single degenerated framework1 (FR1) primer ABL013 (5'-GAGGTBCAR**CTGCAG**GASTCYGG-3'), introducing a *Pst*I restriction site (in bold), in combination with the oligo-dT primer as is described in EP01205100.9. This amplification yields two fragments of 1650 bp and 1300 bp, the latter being the product derived from the CH1-deleted HcAb genes. The smaller PCR-product was gel purified and subsequently digested with *Pst*I and *Bst*EII. The *Bst*EII-site frequently occurs within the FR4 of heavy-chain derived VHH encoding DNA-fragments.
Alternatively, the VHH-repertoire was amplified in a hinge-dependent approach using two IgG specific oligonucleotide primers. In a single PCR reaction a short (5'-AACAGTTAAGCTTCCGCTT**GCGGCCGC**GGAGCTGGGGTCTTCGCTGTGGTGCG-3') or long (5'-AACAGTTAAGCTTCCGCTT**GCGGCCGC**TGGTTGTGGTTTTGGTGTCTTGGGTT-3') hinge primer known to be specific for HcAbs was combined with the FR1-primer ABL013 (see above). A *Pst*I and *Not*I (bold underlined) restriction site was introduced within the FR1 and hinge primers respectively, to allow cloning. Subsequently, the DNA fragments were ligated into *Pst*I-*Bst*EII or *Pst*I-*Not*I digested phagemid vector pAX004, which is identical to pHEN1 (Hoogenboom *et al.*, 1991), but encodes a carboxyterminal $(His)_6$- and c-myc-tag for purification and detection, respectively. The ligation mixture was desalted on a Microcon filter (YM-50, Millipore) and electroporated into *E. coli* TG1 cells to obtain a library containing $1.8x10^7$ clones. The transformed cells were grown overnight at 37°C on a single 20x20 cm plate with LB containing 100 µg/ml ampicillin and 2 % glucose. The colonies were scraped from plates using 2xTY medium and stored at -80°C in 20 % glycerol.

As quality control the percentage of insert containing clones was verified on 24 clones for each library by PCR using a combination of vector based primers. This analysis revealed that 95 % of the clones contained a VHH encoding insert. The variability was examined by *Hin*FI fingerprint analysis of the amplified VHH fragment of these 24 clones, thereby showing that all clones were indeed different (data not shown).

2) Selection of antagonistic anti-TNF VHH's

From both libraries phage was prepared. To rescue the polyclonal phage repertoire, libraries were grown to logarithmic phase (OD600 = 0.5) at 37°C in 2xTY containing 100 µg/ml ampicillin and 2 % glucose and subsequently superinfected with M13K07 helper phage for 30 minutes at 37°C. Infected cells were pelleted for 5 minutes at 4000 rpm and resuspended in 2xTY containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. Bacteriophage was propagated by overnight growth at 37°C and 250 rpm. Overnight cultures were centrifuged for 15 minutes at 4500 rpm and phage was precipitated with one fifth volume of a [20% polyethyleneglycol 6000, 1.5 M NaCl]-solution by incubation for 30 minutes on ice. Phage was pelleted by centrifugation for 15 minutes at 4000xg and 4°C. After resuspension of the phages in PBS, cell debris was pelleted by centrifugation for 1 minute at maximal speed (15000xg) in microcentrifuge tubes. The supernatant containing the phage particles was transferred to a new tube and again phage was precipitated as described above. Phage was dissolved in PBS and separated from remaining cell debris as mentioned above. The titer of phage was determined by infection of logarithmic TG1 cells followed by plating on selective medium.

The library was selected using *in vitro* biotinylated TNF-alpha. The biotinylation was carried out as described by Magni et al (Anal Biochem 2001, 298, 181-188). The incorporation of biotin in TNF was evaluated by SDS-PAGE analysis and detection with Extravidin-alkaline phosphatase conjugate (Sigma). The functionality of the modified protein was evaluated for its ability to bind to the solid phase coated recombinant a p75 receptor.

VHH were selected by capturing biotinylated TNF-alpha (10 to 400 ng per well during 2 hours at room temperature) on streptavidin coated microtiter plates (coated with 100 µl of 10 µg/ml streptavidin during 16 hours at +4°C). Antagonistic VHH were obtained by elution with an excess of receptor, either the extracellular ligand binding domain or with cells expressing the receptor. After 2 hours incubation of phage with captured cytokine, the non-specific phage was washed away, while specific phage displaying antagonistic VHH was eluted for 30 minutes with receptor (extracellular domain of CD120b or p75; 10 µM) or with receptor displaying cells (>10^7 KYM cells per well). High enrichments, i.e. the ratio of the number of phage eluted with receptor and those eluted by serum albumin (50 µg per well), of more than a factor of 20 suggested the successful selection of TNF specific clones. Alternatively, instead of elution with receptor a standard procedure was applied, in which a low pH causes the denaturation of VHH and / or antigen (0.1 M glycine buffer pH

2.5). Log phase growing *E. coli* cells were infected with the eluted and neutralized phage and plated on selective medium.

Individual clones were picked and grown in microtiter plate for the production of VHH in culture supernatant. ELISA screening with TNF captured on Extravidin coated plates revealed about 50% positive clones. *Hin*FI-fingerprint analysis showed that 13 different clones were selected, which were grown and induced on 50 ml scale. The sequences of said clones are shown in Table 2.

Five clones, coded VHH#1, #2, #3, #9 and #13, with different sequences (Figure 15) were characterized in more detail. VHH#2, #3 and #9 are single-domain antibody fragments carrying the typical hydrophilic residue at position 45 (arginine) and the phenylalanine to tryptophan substitution in position 47 in FR2 thereby conferring the advantageous characteristics in terms of solubility. VHH#1 contains the hydrophobic FR2 residues typically found in double-chain antibodies of human origin or from other species, but compensating this loss in hydrophilicity by the charged arginine residue on position 103 that substitutes the conserved tryptophan residue present in VH from double-chain antibodies (PCT/EP02/07804). The new class of Cameloid single-domain antibodies described in this invention is represented by VHH#13, which contains the hydrophobic residues in FR2 in combination with the hydrophobic residue tryptophan at position 103. Larger amounts of antibody fragments were expressed by cultivation on 50 ml scale and purified by IMAC using TALON resin (Clontech). After dialysis against PBS to remove the eluent imidazol the amount of VHH was determined by OD280; approximately 300 µg of VHH was obtained from each clone.

This material was used for determining the sensitivity of detection of (biotinylated) TNF in ELISA. For this purpose a streptavidin (10 µg/ml) coated microtiterplate was employed for capture of biotinylated VHH (1 µg/ml), VHH was diluted in 0.2 % casein / PBS and incubated for 2 hours at room temperature. Bound VHH was detected with anti-MYC mAB 9E10 (0.5 µg/ml) and anti-mouse AP conjugate (1000-fold diluted, Sigma). The results are shown in Figure 16.

3) *Determination of antagonistic effect in cytotoxic assay with KYM cell line*

TNF-induced cytostasis/cytotoxicity was determined by the calorimetric MTT assay as described by Vandenabeele and colleagues (Vandenabeele, P., Declercq, W., Vercammen, D., Van de Craen, M., Grooten, J., Loetscher, H., Brockhaus, M., Lesslauer, W., Fiers, W. (1992) Functional characterization of the human tumor necrosis factor receptor p75 in a transfected rat/mouse T cell hybridoma. J. Exp. Med. 176, 1015-1024.). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is a pale yellow

substrate that is cleaved by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even freshly dead cells do not cleave significant amounts of MTT. KYM cells (Sekiguchi M, Shiroko Y, Suzuki T, Imada M, Miyahara M, Fujii G. (1985) Characterization of a human rhabdomyosarcoma cell strain in tissue culture. Biomed. Pharmacother. 39, 372-380.) were seeded in 96 well microtiterplates and cultured in the presence or absence of TNF-alpha (0.216 ng/ml or approx. 5 pM of trimer). In addition to TNF variable amounts of antibody (VHH or Remicade) were included during cultivation. For the assay MTT was added to the culture medium at a final concentration of 500 µg/ml and the plates were incubated at 37°C to achieve cleavage of MTT by mitochondrial enzymes. The formed formazon product, which appear as black, fuzzy crystals on the bottom of the well are dissolved by addition of acid isopropanol (40 nM HCl in isopropanol) or DMSO. The absorbance is measured at 570 nm.

The MTT assays (Figure 17) show that VHH#13, representing the new class of VHH, has good antagonistic characteristics, even better than VHH#1, which has arginine on position 103 in combination with the human-like hydrophobic residues in FR2. VHH#9 with the characteristic hydrophilic residues in FR2 does not prevent binding of TNF-α to its ligand in spite of its sensitive detection of the cytokine in ELISA. In contrast, VHH#2 and #3 with hydrophilic FR2 hallmark residues are very potent antagonistic VHH's. VHH#2 and #3 have a high degree of homology and are clonally related (Harmsen et al., Mol. Immunol. 37, 579-590), but only VHH#2 is as potent as Remicade, probably due to the fact that it has a higher affinity than VHH#3 (Figure 16).
These experiments show that the new class of VHH has bona fide binding and functional characteristics, thereby enabling their application for therapeutic purposes.

Example 15: Experimental evidence that C37 belongs to the new class of VHHs

A PCR was performed on total cDNA prepared from llama002, with a primer specific for the CDR3 of C37 (5' GGGGCCGGTACTAGTTCATACTTGCC 3') and a primer in the CH2 domain (5' GGAACAGTTCAACAGCACGTACC 3') (Figure 18). A fragment of 360 bp was obtained (Figure 19), indicating that the CH1 domain is not present thus meaning that this VHH is derived from the heavy chain subclass.

CLAIMS

1. A polypeptide derived from a *Camelidae* species single domain heavy chain antibody comprising at position 103 a tryptophan residue and at position 45 an amino acid residue selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tyrosine, tryptophan, methionine, serine, threonine, asparagine, glutamine, said positions determined according to the Kabat numbering, said antibody directed to an antigen.

2. A polypeptide according to claim 1 consisting of a sequence corresponding to SEQ ID NO: 1 or SEQ ID NO: 13.

3. A polypeptide derived from a *Camelidae* species single domain heavy chain antibody directed against a Tumor Necrosis Factor alpha (TNF-alpha) peptide antigen.

4. A polypeptide according to claim 3 consisting of a sequence corresponding to any of SEQ ID NOs: 11 to 23.

5. A polypeptide derived from a *Camelidae* species single domain heavy chain antibody directed against a von Willebrand Factor (vWF) peptide antigen, a vWF A1 domain peptide antigen, a vWF A3 domain peptide antigen , a glycoprotein Ib peptide antigen, a glycoprotein Ia/IIa peptide antigen or a collagen peptide antigen.

6. A polypeptide according to claim 5 consisting of a sequence corresponding to any of SEQ ID NOs: 1 to 9.

7. A polypeptide according any of claims 1 to 6 which is a humanised polypeptide.

8. A polypeptide comprising a polypeptide according to any of claims 1 to 7, or an homologous sequence of a polypeptide according to any of claims 1 to 7, or a portion of an homologous sequence of a polypeptide according to any of claims 1 to 7.

9. A nucleic acid encoding a polypeptide of any of claims 1 to 8.

10. A polypeptide according to any of claims 1 to 8 or a nucleic acid according to claim 9 for use as a medicine.

11. A method of identifying an agent that modulates the binding of a polypeptide of claims 1, 2, 7 and 8 to said antigen comprising:

(a) contacting a polypeptide according to claims 1, 2, 7 and 8 with said antigen, a homologous sequence of said antigen, and/or a functional portion of said antigen, in the presence and absence of a candidate modulator under conditions permitting binding between said polypeptide and antigen, and

(b) measuring the binding between the polypeptide and antigen of step (a), wherein a decrease in binding in the presence of said candidate modulator, relative to the binding in the absence of said candidate modulator identified said candidate modulator as an agent that modulates the binding of a polypeptide of claims 1, 2, 7 and 8 and said antigen.

12. A method of identifying an agent that modulates TNF-alpha-mediated disorders through the binding of a polypeptide of claims 3, 4, 7 and 8 to TNF-alpha comprising

(a) contacting a polypeptide according to claims 3, 4, 7 and 8 with said antigen, a homologous sequence of said antigen, and/or a functional portion of said antigen, in the presence and absence of a candidate modulator under conditions permitting binding between said polypeptide and antigen, and

(b) measuring the binding between the polypeptide and antigen of step (a), wherein a decrease in binding in the presence of said candidate modulator, relative to the binding in the absence of said candidate modulator identified said candidate modulator as an agent that modulates TNF-alpha-mediated disorders.

13. A method of identifying an agent that modulates the binding of TNF-alpha to its receptor through the binding of a polypeptide of claims 3, 4, 7 and 8 to TNF-alpha comprising

(a) contacting a polypeptide according to claims 3, 4, 7 and 8 with TNF-alpha, a homologous sequence of TNF-alpha, or a functional portion of TNF-alpha, in the presence and absence of a candidate modulator under conditions permitting binding between said polypeptide and TNF-alpha, and

(b) measuring the binding between the polypeptide and TNF-alpha of step (a), wherein a decrease in binding in the presence of said candidate modulator, relative to the binding in the absence of said candidate modulator identified said candidate modulator as an agent that modulates the binding of TNF-alpha to its receptor.

14. A method of identifying an agent that modulates platelet aggregation through the binding of a polypeptide of claims 5 to 8 and said antigen comprising

(a) contacting a polypeptide according to claims 5 to 8 with said antigen, a homologous sequence of said antigen, and/or a functional portion of said antigen, in the presence and absence of a candidate modulator under conditions permitting binding between said polypeptide and antigen, and

(b) measuring the binding between the polypeptide and antigen of step (a), wherein a decrease in binding in the presence of said candidate modulator, relative to the binding in the absence of said candidate modulator identified said candidate modulator as an agent that modulates platelet aggregation.

15. A kit for screening for agents that modulate polypeptide-antigen binding according to the method of claim 11.

16. A kit for screening for agents that modulate TNF-alpha-mediated disorders according to the methods of claims 12 and 13.

17. A kit for screening for agents that modulate platelet aggregation according to the method of claim 14.

18. An unknown agent that modulates the binding of the polypeptides of claims 1, 2, 7 and 8 to said antigen, identified according to the method of claim 11.

19. An unknown agent that modulates TNF-alpha-mediated disorders, identified according to the methods of claims 12 and 13.

20. An unknown agent that modulates platelet aggregation identified according to the method of claim 14.

21. Use of a polypeptide according to any of claims 1, 2, 7 and 8, a nucleic acid according to claim 9, or an agent according to claim 18 for the preparation of a medicament for treating and/or preventing and/or alleviating conditions relating to immune and inflammatory reactions.

22. Use of a polypeptide according to any of claims 1, 2, 7 and 8, a nucleic acid according to claim 9, or an agent according to claim 18 for the treatment of a condition relating to immune and inflammatory reactions.

23. Use of a polypeptide according to any of claims 1, 2, 7 and 8, a nucleic acid according to claim 9, or an agent according to claim 18 for the preparation of a medicament for treating and/or preventing and/or alleviating symptoms of inflammatory diseases.

24. Use of a polypeptide according to any of claims 1, 2, 7 and 8, a nucleic acid according to claim 9, or an agent according to claim 18 for the preparation of a medicament for the treatment of a condition relating to immune and inflammatory reactions.

25. Use of a polypeptide according to any of claims 3, 4, 7 and 8, a nucleic acid according to claim 9, or an agent according to claim 19 for the preparation of a medicament for treating and/or preventing and/or alleviating conditions relating to one or more or rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel disease and multiple sclerosis.

26. Use of a polypeptide according to any of claims 5 to 8, a nucleic acid according to claim 9 or an agent according to claim 20 for the preparation of a medicament for treating and/or preventing and/or alleviating conditions relating to platelet aggregation or dysfunction thereof.

27. Use of a polypeptide according to any of claims 5 to 8, a nucleic acid according to claim 9 or an agent according to claim 20 for the preparation of a medicament for treating and/or preventing and/or alleviating conditions arising from one or more of transient cerebral ischemic attack, unstable angina pectoris, cerebral infarction, myocardial infarction, peripheral arterial occlusive disease, restenosis, coronary by-pass graft, or coronary artery valve replacement and coronary interventions such angioplasty, stenting, or atherectomy.

28. Use of a polypeptide according to any of claims 5 to 8, a nucleic acid according to claim 9 or an agent according to claim 20 for the preparation of a medicament for the prevention of the formation of a non-occlusive thrombus.

29. Use of a polypeptide according to any of claims 5 to 8, a nucleic acid according to claim 9 or an agent according to claim 20 for the preparation of a medicament for the prevention of the formation of an occlusive thrombus.

30. Use of a polypeptide according to any of claims 5 to 8, a nucleic acid according to claim 9 or an agent according to claim 20 for the preparation of a medicament for the prevention of arterial thrombus formation.

31. Use of a polypeptide according to any of claims 5 to 8, a nucleic acid according to claim 9 or an agent according to claim 20 for the preparation of a medicament for the prevention of acute coronary occlusion.

32. Use of a polypeptide according to any of claims 5 to 8, a nucleic acid according to claim 9 or an agent according to claim 20 for the preparation of a medicament for maintaining the patency of diseased arteries.

33. Use of a polypeptide according to any of claims 5 to 8, a nucleic acid according to claim 9 or an agent according to claim 20 for the preparation of a medicament for the prevention of restenosis.

34. Use of a polypeptide according to any of claims 5 to 8, a nucleic acid according to claim 9 or an agent according to claim 20 for the preparation of a medicament for the prevention of restenosis after PCTA or stenting.

35. A polypeptide according to any of claims 5 to 8, a nucleic acid according to claim 9 or an agent according to claim 20 for the preparation of a medicament for the prevention of thrombus formation in stenosed arteries.

36. Use of a polypeptide according to any of claims 5 to 8, a nucleic acid according to claim 9 or an agent according to claim 20 for the preparation of a medicament for the prevention of hyperplasia after angioplasty, atherectomy or arterial stenting

37. Use of a polypeptide according to any of claims 5 to 8, a nucleic acid according to claim 9 or an agent according to claim 20 for the preparation of a medicament for the prevention of unstable angina.

38. Use of a polypeptide according to any of claims 5 to 8, a nucleic acid according to claim 9 or an agent according to claim 20 for the preparation of a medicament for the prevention or treatment of occlusive syndrome in a vascular system.

39. A method of diagnosing a disease or disorder characterised by a dysfunction of a polypeptide comprising the antigen of claims 1, 2, 7 and 8 comprising:

(a) contacting a sample with a polypeptide according to any of claims 1, 2, 7 and 8, and

(b) detecting binding of said polypeptide to said sample, and

(c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disease or disorder characterised by dysfunction of the antigen of claims 1, 2, 7 and 8.

40. A method of diagnosing a disease or disorder characterised by the dysfunction of TNF-alpha comprising:

(a) contacting a sample with a polypeptide according to any of claims 3, 4, 7 and 8, and

(b) detecting binding of said polypeptide to said sample, and

(c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disease or disorder characterised by dysfunction of TNF-alpha.

41. A method of diagnosing a disease or disorder characterised dysfunction of platelet aggregation comprising:

(a) contacting a sample with a polypeptide according to any of claims 5 to 8, and

(b) detecting binding of said polypeptide to said sample, and

(c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disease or disorder characterised by dysfunction of platelet aggregation.

42. A kit for screening for a disease or disorder of claims 39 to 41, using the methods according to claims 39 to 41 respectively.

43. A kit according to claim 42 comprising an isolated polypeptide according to any of claims 1 to 8.

44. Use of a polypeptide according to any of claims 1, 2, 7 and 8 for the purification of said antigen.

45. Use of a polypeptide according to any of claims 3, 4, 7 and 8 for the purification TNF-alpha.

46. Use of a polypeptide according to any of claims 5 to 8 for the purification of von Willebrand factor, von Willebrand factor A1 domain, von Willebrand factor A3 domain, gpIb, gpIa/IIa, or collagen type I.

47. Use of a polypeptide of any of claims 3, 4, 7 and 8 for inhibiting the interaction between TNF-alpha and one or more TNF-alpha receptors.

48. Use of a polypeptide of any of claims 5 to 8 for inhibiting the interaction between von Willebrand factor and collagen and/or platelet receptors.

49. Use of a polypeptide of any of claims 5 to 8 for inhibiting platelet aggregation.

50. Use of a polypeptide according to any of claims 1 to 8 or a nucleic acid of claim 9 for the manufacture of a medicament to be administered orally, for the prevention or treatment of any of the disorders of claims 21 to 38.

51. A method for producing a polypeptide according to claim 1 comprising the steps of:
(a) obtaining double stranded DNA encoding a camelidae species single domain heavy chain antibody directed to said antigen,
(b) selecting and screening DNA comprising at position 103 a tryptophan residue and at position 45 an amino acid residue selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tyrosine, tryptophan, methionine, serine, threonine, asparagine, glutamine, and
(c) cloning and expressing the DNA selected in step (b).

52. A method of producing a polypeptide according to any of claims 1 to 8 comprising
(a) culturing host cells comprising nucleic acid capable of encoding a polypeptide according to any of claims 1 to 8, under conditions allowing the expression of the polypeptide, and,
(b) recovering the produced polypeptide from the culture.

53. A method according to claim 52, wherein said host cells are bacterial or yeast.

54. A method for humanising a Camelidae antyibody or antibody fragment comprising introducing in the amino acid sequence of said antibody or antibody fragment, at position

103 a tryptophan residue and at position 45 an amino acid residue selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tyrosine, tryptophan, methionine, serine, threonine, asparagine, glutamine said positions determined according to the Kabat numbering.

55. A method for humanising a Camelidae antibody or antibody fragment comprising introducing in the nucleic acid sequence of said antibody or antibody fragment, at amino acid position 103 a codon encoding a tryptophan residue and at amino acid position 45 a codon encoding an amino acid residue selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tyrosine, tryptophan, methionine, serine, threonine, asparagine, glutamine said positions determined according to the Kabat numbering.

ABSTRACT

The present invention relates to polypeptides derived from Camelidae single domain heavy chain antibodies directed to tumor necrosis factor alpha and von Willebrand factor. It further relates to a new class of a camelid single domain heavy chain antibodies (VHHs) that have human-like sequences. It further relates to methods of administering therapeutic polypeptides.

FIGURE 1

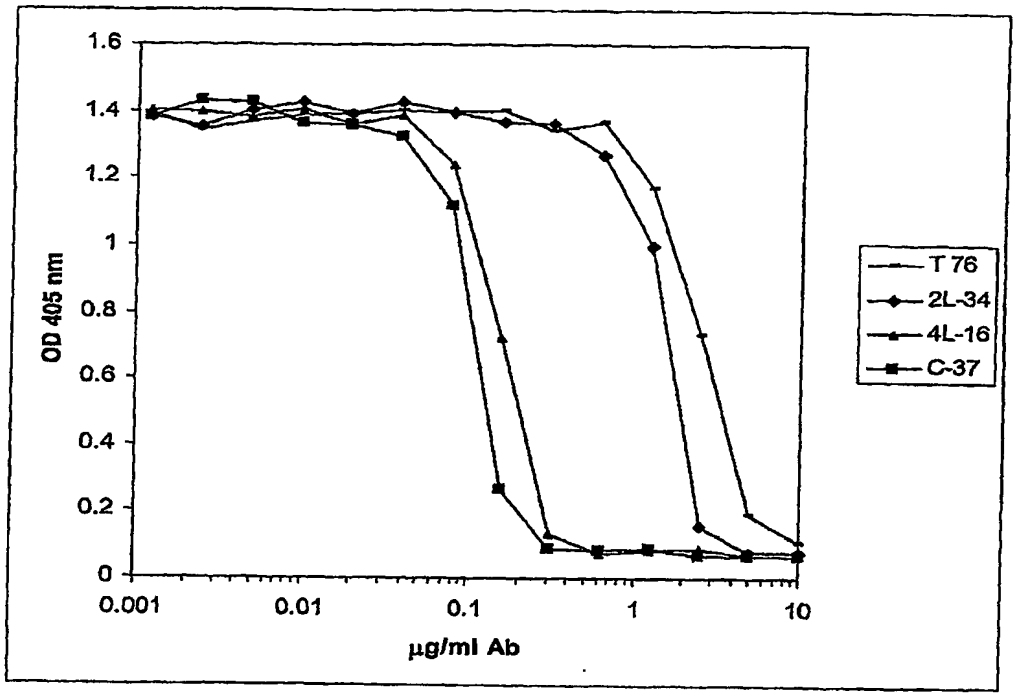

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD 405 nm
0.001
0.01
0.1
1
10
µg/ml Ab
T 76
2L-34
4L-16
C-37

FIGURE 2

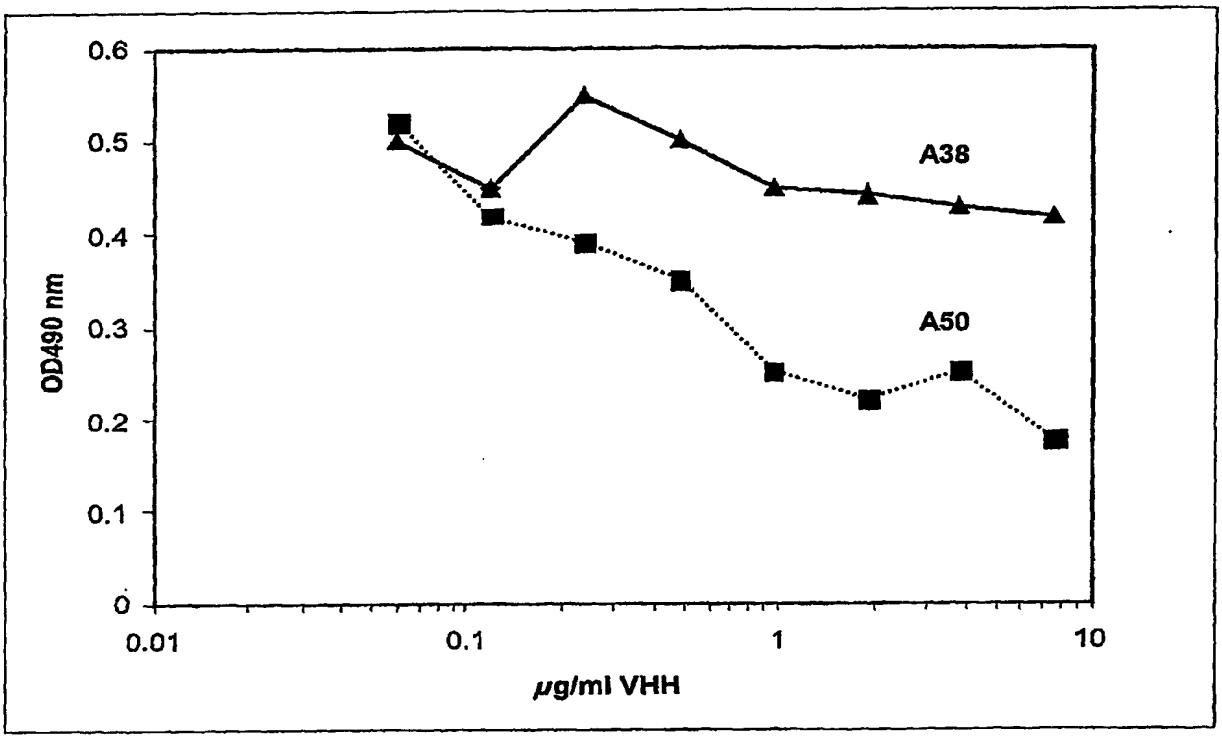
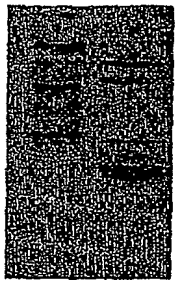
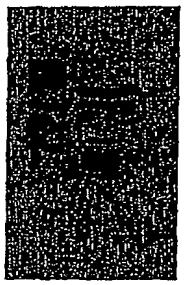


FIGURE 3



A1 domain



A3 domain

FIGURE 4

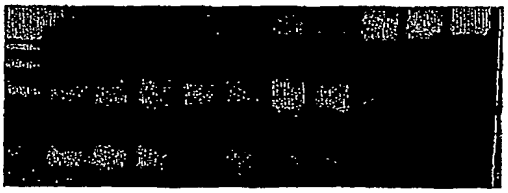


FIGURE 5

Sequences A1 domain specific binders:

```
        --------framework 1---------CDR1---framework 2-----CDR2----
A50     LQESGGGLVQAGGSLRLSCAASGRTFS SYRMG WFRQAPGKEREFVA AISRRGDNVY
A38     LQDSGGGSVQAGGSLRLSCAASGRTVS SYNMG WFRRVPGKERDFVA AISWSGVATY
I53     LQDSGGGLVQAGGSLRLSCAASGRTKD MA    WFRQPPGKEREFVA VIYSSDGSTL
M53     LQDSGGGLVQAGESLRLSCGTSGRTFG RRAMA WFRQAPGKERQFVA WIARYDGSTL
Z29     LQESGGGSVQAGDSLTLSCAASGRTFS MHAMG WFRQAPGKEREFVA AISPSAFTTE

        ------framework 3----------------------CDR3---------
A50     YADSVKGRFAISRDNAESTLYLQMNSLKPEDTAVYYCAA HVTVSAITLSTSTYDY
A38     YFDSVKGRFTISRDNAKNTVYLEMNSLKPEDTAVYYCAA ASRYRHRLNSGSEYDY
I53     VAASVKGRFTISRDNAKNTVYLQMTSLKPADTAVYYCAT SRGYSGTYYSTSRYDY
M53     YADSVKGRFTISRDDNKNTMYLHMNNLTPEDTAVYYCAA   GPRGLYY ESRYEY
Z29     YADSLKGRFTVSRDNAKKLVWLQMNGLKPEDTAAYYCAA RRGAFTATTAP LYDY

        framework4-
A50     WGQGTQVTVSS
A38     WGQGTQVTVSS
I53     WTGGTQVTVSS
M53     WGQGTLVTVSS
Z29     WGQGTQVTVSS
```

Sequence of A3 domain specific binders:

```
        --------framework 1---------CDR1---framework 2-----CDR2----
C37     LQESGGGLVQPGGSLRLSCAASGFNFN WYPMS WVRQAPGKGLEWVS TISTYGEPR
T76     LQESGGGLVQPGESLRLSCAASGSIFS INTMG WYGQAPGKQRELVA SITFGGVTN
2L-34   LQDSGGGLVQAGGSLRLSCAASVRIFT SYAMG WFRQAPGKEREFVA AINRSGKSTY
4L-16   LVESGGGLVQAGGSLRLSCAASGRTFS SYAMG WFRQAPGKEREFVA AISWSGGSTY

        ------framework 3----------------------CDR3---------
C37     YADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYYCAR GAGTSSYLPQRGN
T76     YADSVKGRFTISRDNTNDTVYLQMNSLKPEDTAVYICNA VTWGGLTNY
2L-34   YSDSVEGRFTISRDNAKNTVSLQMDSLKLEDTAVYYCAA DYSGSYTSLWSRPERLD
4L-16   YADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCVA DTGGISWIRTQGYNY

        framework4-
C37     WDQGTQVTISS
T76     WGQGTQVTVSS
2L-34   WGQGTQVTVFS
4L-16   WGQGTQVTVSS
```

FIGURE 6

GEGLQIPTLSPAPDCSQPLDVILLLDGSSSFPASYFDEMKSFAKAFISKANIGPRLT
QVSVLQYGSITTIDVPWNVVPEKAHLLSLVDVMQREGGPSQIGDALGFAVRYLTSEM
HGARPGASKAVVILVTDVSVDSVDAAADAARSNRVTVFPIGIGDRYDAAQLRILAGP
AGDSNVVKLQRIEDLPTMVTLGNSFLHKLCSGF

FIGURE 7

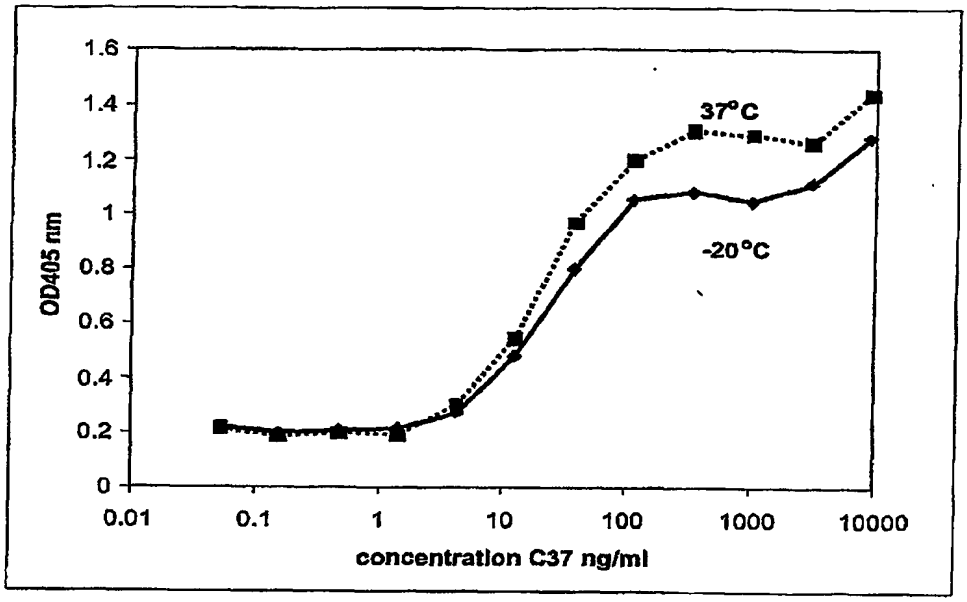


FIGURE 8

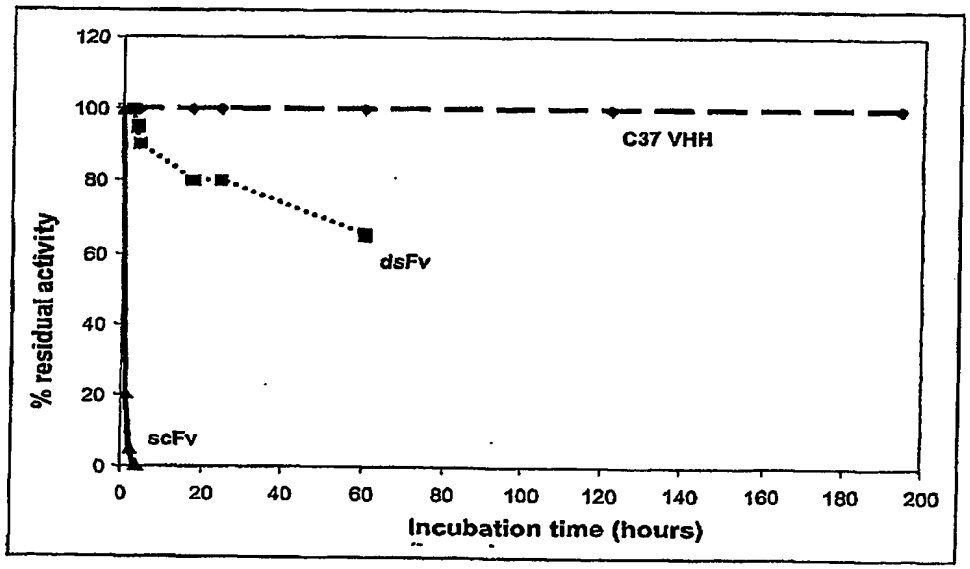


FIGURE 9

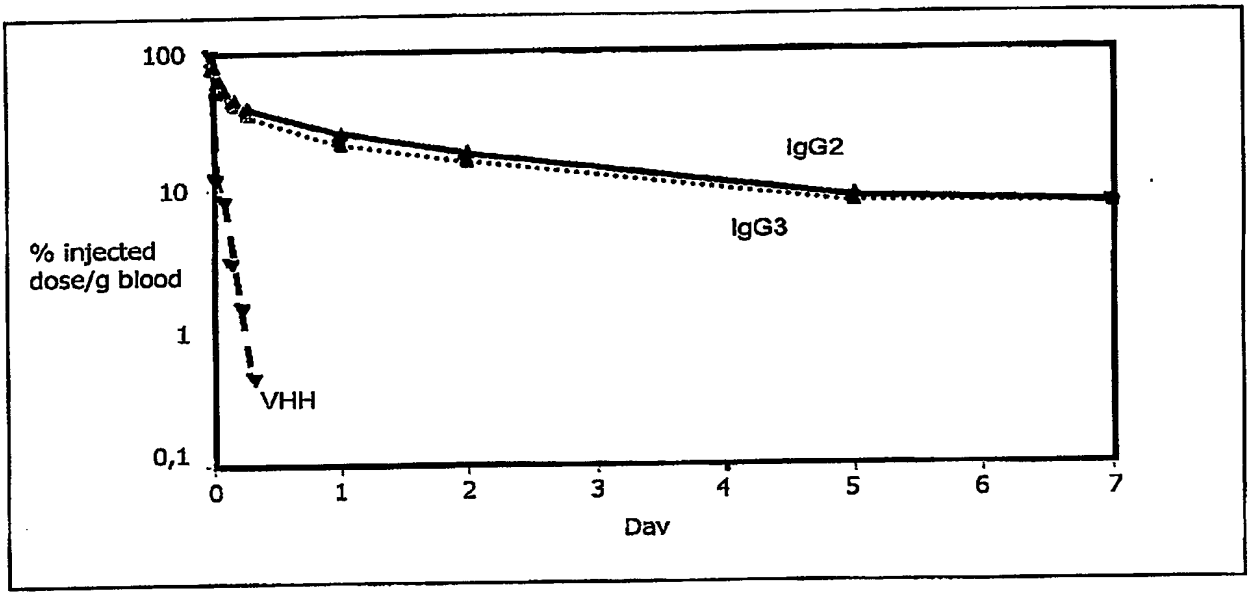


FIGURE 10

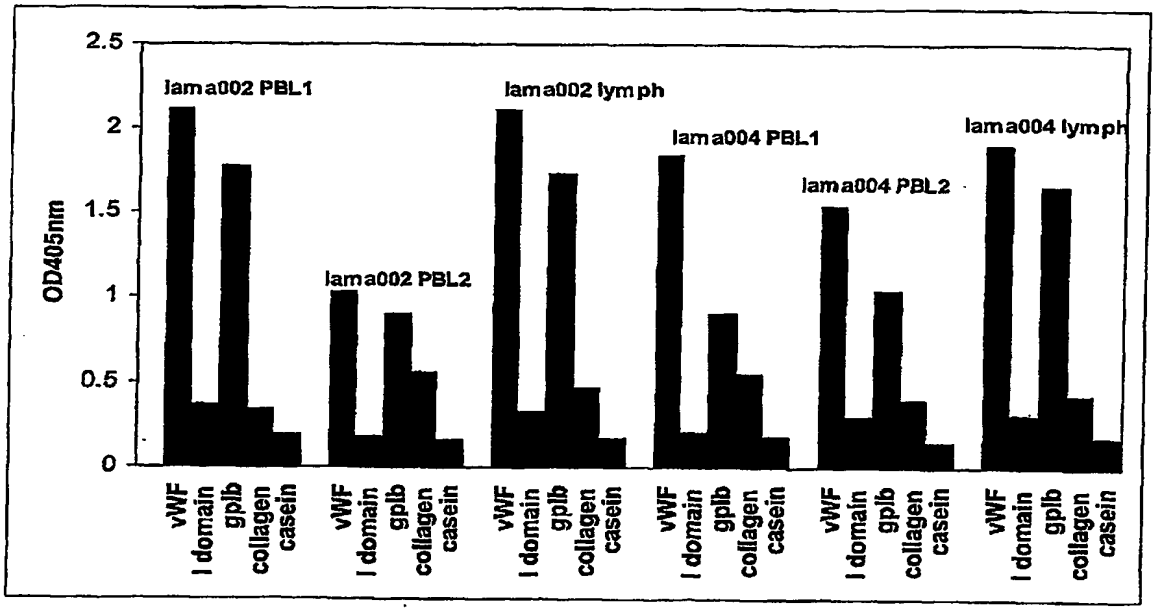
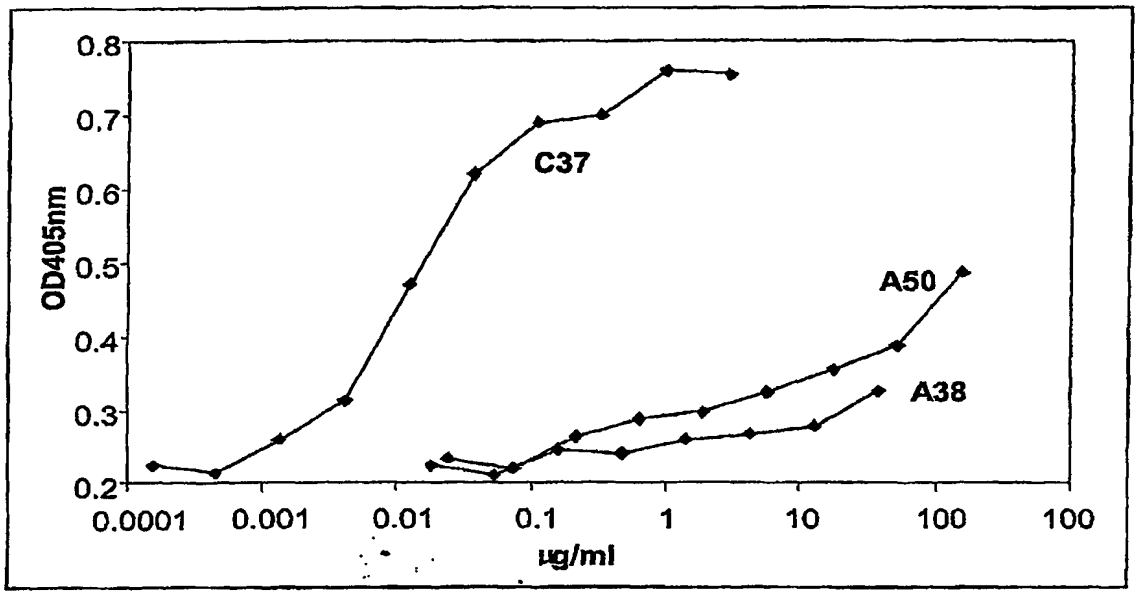

2.5
2
1.5
1
0.5
0
OD405nm
lama002 PBL1
lama002 PBL2
lama002 lymph
lama004 PBL1
lama004 PBL2
lama004 lymph
vWF
I domain
gpIb
collagen
casein

FIGURE 11


0.8
0.7
0.6
0.5
0.4
0.3
0.2
OD405nm
C37
A50
A38
0.0001
0.001
0.01
0.1
1
10
100
100
µg/ml

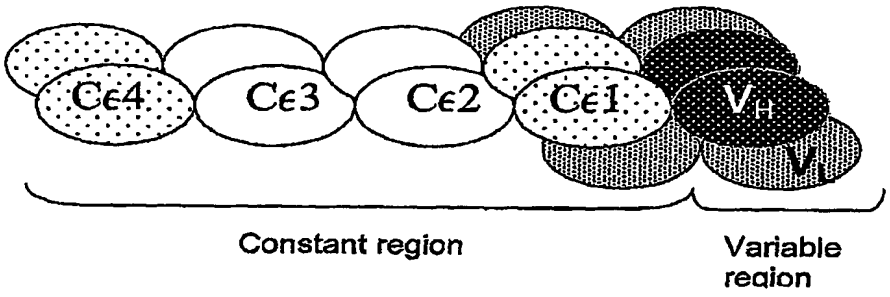


FIGURE 12

SEQ ID NO	NAME	SEQUENCE
24	EV	QVQLQDSGGGLVQPGGSLRLSCRASGRIFRINAMGWYRQAPGKQRELVATITSTGSTNF
	2C3	ADSVKGRFTIYRDGAKRTVDLRLNSLKPEDTAVYFCNADVREYDLGPWRQYWGQGTQVTVSS
25	EV	QVQLQESGGGVVQPGGSLRLSCSVSGTSISNRVMAWFRQAPGKQRDFVAYITSAVNTDY
	4G12	ADFVKGRFTISRDNAQNMVHLQMNSLKPEDTAVYYCNVLKDTWFRTPYDYYWGQGTQVTVSS
26	EV	QVQLQESGGGLVQPGDSLRLSCVVSGRTLSYSSLAWFRQAPGKERDFVAALSLTTYY
	2C1	ADSVKGRFTISRDNAKNTVYLQMNSLKPDDTADYFCATARTRTDYAPLLSAASTYDAWGQGTQVTVSL
27	EV	QVQLQESGGGLVQAGGSLRLSCAASGRSSRYYAMGWFRQGPGKEREFVAAVNWNGDSTYY
	2H3	ADSVKGRFTISRGNAENTAYLQMNSLVPEDTAVYYCAMRMNAGLGYSAASYQYWGQGTQVTVSL
28	EV	QVQLQESGGGLVQAGDSLRLSCAASGLTFLEHVMAWFRQTPGKEREFVGAIDWSGRRITY
	2D12	TDSVKGRFTISRDNAKNTVYLQMNTLKPEDTAVYYCAADRTYSYSSTGYYYWGQGTQVTVSS
29	EV	QVQLQDSGGGLVQAGDSLRLSCAASGLTFLEHVMAWFRQTPGKEREFVGAIDWSGRRITY
	2G4	TDSVKGRFTISRDNAKNTVYLQMNTLKPEDTAVYYCAADRTYSYSSTGYYYWGQGTQVTVSS
30	EV	QVQLQESGGGLVQAGGSLRLSCAASGRTLSSYTMAWFRQAPGKEREFVASISSSGISTYY
	4C5	ADSVKGRFTISRDIAKNTVYLQMNSLKPEDTAVYYCAAKYRYYSTLYTKSGEYDYWGQGTQVTVSS
31	EV	QVQLQDSGGGLVQAGGSLRLSCEASGRTISSYAMAWFRQAPGKEREFVASISSSGVSKHY
	4A2	ADSVKGRFTISNDKVKNTVYLQMNSLKPEDTAVYFCAAKYRYYSSYYTKSGDYDYWGQGTQVTVSS
32	EV	QVQLQESGGGLVQAGGSLRLSCAASGLTFSTYAMGWFRQAPGKEREFVAAVSYSGSYY
	2D4	ADSVKGRFTISRDNAKNTVYLQMASLKPEDTAVYYCAARNRGYSTYAGVYDYWGQGTQVTVSS
33	EV	QVQLQDSGGGLVQAGGSLRLSCAASGVTFSSYAMGWFRQAPGKEREFVASITWIGGGTYY
	2B6	ADSVKGRFTISRDHAGNTVYLQMNTLKPDDTAVYYCALDRRSSTYYLMKGEYDYRGRGTQVTVSS
34	EV	QVQLQESGGGLVQAGGSLRLSCAASGVTFSSYAMGWFRQAPGKEREFVASITWTGTGTYY
	2H11	ADSVKGRFTISRDHAGTTVYLQMNSLKPEDTAVYYCAVDRRSSTYYLMKGEYDYRGRGTQVTVSS

FIGURE 13

```
EV 2C3    QVQLQDSGGGLVQPGGSLRLSCRASGRIFRINAMGWYRQAPGKQRELVATITSTG-STNF
EV 4G12   QVQLQESGGGVVQPGGSLRLSCSVSGTSISNRVMAWFRQAPGKQRDFVAYIT-SAVNTDY
EV 2C1    QVQLQESGGGLVQPGDSLRLSCVVSGRTLSYSSLAWFRQAPGKERDFVAALSLT---TYY
EV 2H3    QVQLQESGGGLVQAGGSLRLSCAASGRSSRYYAMGWFRQGPGKEREFVAAVNWNGDSTYY
EV 2D12   QVQLQESGGGLVQAGDSLRLSCAASGLTFLEHVMAWFRQTPGKEREFVGAIDWSGRRITY
EV 2G4    QVQLQDSGGGLVQAGDSLRLSCAASGLTFLEHVMAWFRQTPGKEREFVGAIDWSGRRITY
EV 4C5    QVQLQESGGGLVQAGGSLRLSCAASGRTLSSYTMAWFRQAPGKEREFVASISSSGISTYY
EV 4A2    QVQLQDSGGGLVQAGGSLRLSCEASGRTISSYAMAWFRQAPGKEREFVASISSSGVSKHY
EV 2D4    QVQLQESGGGLVQAGGSLRLSCAASGLTFSTYAMGWFRQAPGKEREFVAAVSYSG--SYY
EV 2B6    QVQLQDSGGGLVQAGGSLRLSCAASGVTFSSYAMGWFRQAPGKEREFVASITWIGGGTYY
EV 2H11   QVQLQESGGGLVQAGGSLRLSCAASGVTFSSYAMGWFRQAPGKEREFVASITWTGTGTYY

EV 2C3    ADSVKGRFTIYRDGAKRTVDLRLNSLKPEDTAVYFCNADVRE------YDLGPWRQYWGQGTQVTVSS
EV 4G12   ADFVKGRFTISRDNAQNMVHLQMNSLKPEDTAVYYC-----NVLKDTWFRTPYDY-YWGQGTQVTVSS
EV 2C1    ADSVKGRFTISRDNAKNTVYLQMNSLKPDDTADYFCATARTRTDYAPLLSAASTYDAWGQGTQVTVSL
EV 2H3    ADSVKGRFTISRGNAENTAYLQMNSLVPEDTAVYYCAMRMNA--GLGY--SAASYQYWGQGTQVTVSL
EV 2D12   TDSVKGRFTISRDNAKNTVYLQMNTLKPEDTAVYYCAAD-RT--YS--YSSTGYY-YWGQGTQVTVSS
EV 2G4    TDSVKGRFTISRDNAKNTVYLQMNTLKPEDTAVYYCAAD-RT--YS--YSSTGYY-YWGQGTQVTVSS
EV 4C5    ADSVKGRFTISRDIAKNTVYLQMNSLKPEDTAVYYCAAKYRY--YSTLYTKSGEYDYWGQGTQVTVSS
EV 4A2    ADSVKGRFTISNDKVKNTVYLQMNSLKPEDTAVYFCAAKYRY--YSSYYTKSGDYDYWGQGTQVTVSS
EV 2D4    ADSVKGRFTISRDNAKNTVYLQMASLKPEDTAVYYCAARNRG--YS---TYAGVYDYWGQGTQVTVSS
EV 2B6    ADSVKGRFTISRDHAGNTVYLQMNTLKPDDTAVYYCALD-RR--SSTYYLMKGEYDYRGRGTQVTVSS
EV 2H11   ADSVKGRFTISRDHAGTTVYLQMNSLKPEDTAVYYCAVD-RR--SSTYYLMKGEYDYRGRGTQVTVSS
```

FIGURE 14

```
                        FR1                          CDR1          FR2
VHH#3   QVQLQDSGGGLVQAGGSLRLSCAVSGR TFSARS--VYTMG WFRQAPGKEREFVA
VHH#2   QVQLQESGGGLVQPGGSLRLSCAASGR TFSDHSGYTYTIG WFRQAPGKEREFVA
VHH#1   QVQLQESGGGLVQPGGSLRLSCATSGF DFS--VSWMY--- WVRQAPGKGLEWVS
VHH#13  QVQLQESGGGLVQPGGSLRLSCATSGF TFS--DYWMY--- WVRQAPGKGLEWVS
VHH#9   QVQLQESGGGLVQPGGSLRLSCAASGS IFRVNA-----MG WYRQVPGNQREFVA
        ***** *******. ********* **    *           * ** **    * *

            CDR2                          FR2
VHH#3   RIYWSSANTYYADSVKG RFTISRDNAKNTVDLLMNSLKPEDTAVYYCAA
VHH#2   RIYWSSGNTYYADSVKG RFAISRDIAKNTVDLTMNNLEPEDTAVYYCAA
VHH#1   EINTNGLITKYVDSVKG RFTISRDNAKNTLYLQMDSLIPEDTALYYCAR
VHH#13  TVNTNGLITRYADSVKG RFTISRDNAKYTLYLQMNSLKSEDTAVYYCTK
VHH#9   -IITSGDNLNYADAVKG RFTISTDNVKKTVYLQMNVLKPEDTAVYYCNA
                  * * *** ** ** *  * *  * *  *  **** ***

            CDR3          FR4       Hinge
VHH#3   RDGIPTSRTVGSYNY WGQGTQVTVSS EPKTPKPQP
VHH#2   RDGIPTSRSVESYNY WGQGTQVTVSS EPKTPKPQP
VHH#1   ---------SPSGSF RGQGTQVTVSS EPKTPKPQP
VHH#13  -VVPPYSDDSRTNAD WGQGTQVTVSS EPKTPKPQP
VHH#9   --ILQTSRWSIPSNY WGQGTQVTVSS EPKTPKPQP
                        ********** *********
```

FIGURE 15

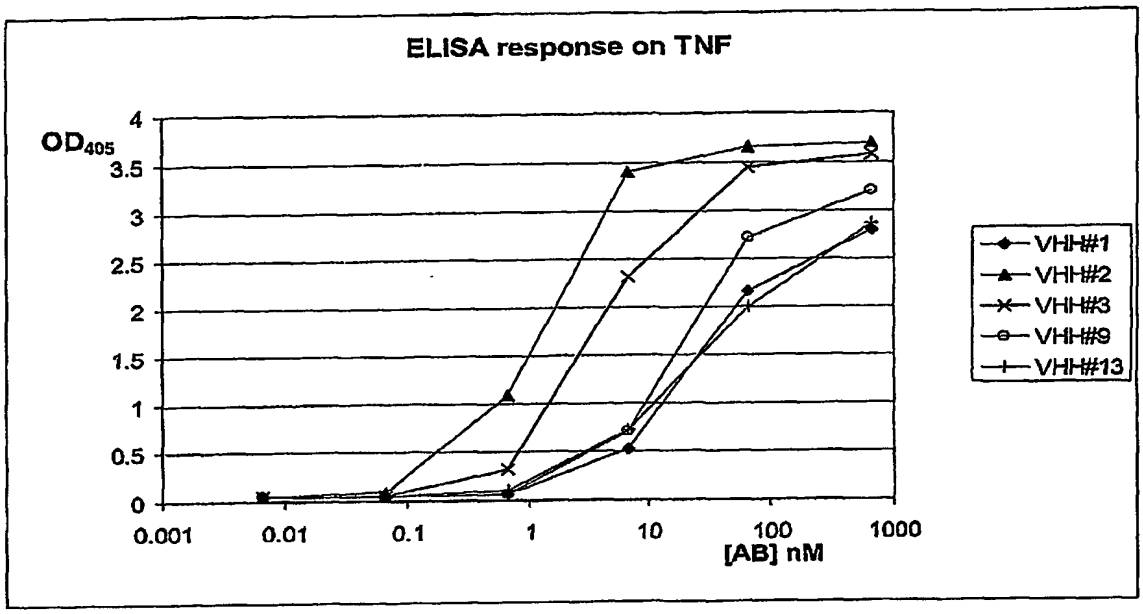


FIGURE 16

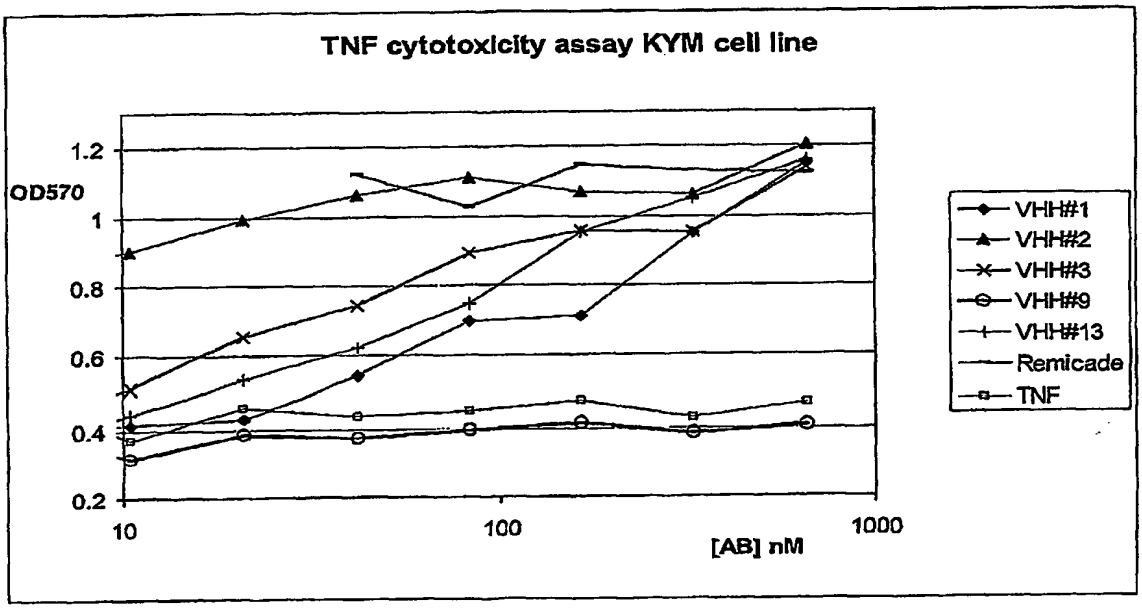


FIGURE 17

FIGURE 18

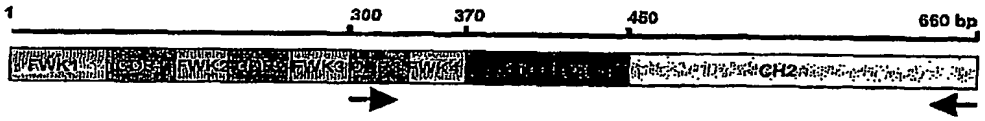


FIGURE 19